mGluR5 Positive Allosteric Modulation as a Novel Therapeutic Target for the Cognitive Deficits Associated with Schizophrenia

by

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ABSTRACT

Patients with schizophrenia have impaired cognitive flexibility, as evidenced by behaviors of perseveration. Cognitive impairments may be due to dysregulation of glutamate and/or loss of neuronal plasticity in the medial prefrontal cortex (mPFC). The purpose of these studies was to examine the effects of mGluR5 positive allosteric modulators (PAMs) alone and in combination with the NMDAR antagonist MK-801, a pharmacological model of schizophrenia. An operant-based cognitive set-shifting task was utilized to assess cognitive flexibility, in vivo microdialysis procedures to measure extracellular glutamate levels in the mPFC, and diolistic labeling to assess the effects on dendritic spine density and morphology in the mPFC. Results revealed that chronic administration of the mGluR5 PAM CDPPB was able to significantly reduce the effects of chronically administered MK-801 on both behavioral perseveration and glutamate neurotransmission. Results also showed that CDPPB had no evidence of an effect on dendritic spine density or morphology, but the mGluR5 negative allosteric modulator fenobam caused significant increases in spine density and the frequency of occurrence of spines with smaller head diameters. Conclusions include that CDPPB is able to reverse MK-801 induced cognitive deficits as well as alterations in mPFC glutamate neurochemistry. The culmination of these studies add further support for targeting mGluR5 with PAMs as a novel mechanism to alleviate cognitive impairments in patients with schizophrenia.

DEDICATION

I would like to dedicate my dissertation to several people: my dad, Brian LaCrosse, my mom, Margaret LaCrosse, my sisters: Crystal Martin, Deanna Fryczynski and Jenny Koivisto, and my boyfriend, Jacob Colantonio. My parents and sisters have provided me with a lifetime of love and support, which gave me the confidence to take on an endeavor as great as earning a doctoral degree. For as long as I can remember, there's never been anything that I didn't think I could accomplish if I gave it my all. I owe that faith in myself entirely to them, because their faith in me never waivered. Finally, the love and support I receive from Jacob Colantonio has sustained me every day throughout the completion of this dissertation and I have no doubt it will sustain me for the rest of my life.

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INTRODUCTION

Background and Significance

Schizophrenia is a long term, progressive, and complex disease. Schizophrenia is comprised of several diverse symptoms that are generally grouped into two categories: positive symptoms and negative symptoms (Basso et al., 1998). Positive symptoms are termed to reflect the addition of behaviors to normal function, and negative symptoms are termed to reflect the loss of behaviors from normal function (Jackson, 1887). Positive symptoms are generally the most prominent behaviors as they typically initiate diagnosis and treatment as these symptoms are clear indicators of a severe psychotic disorder (Andreasen, 1995). Positive symptoms include disorders of perception (hallucinations, usually auditory in nature), inferential thinking (persistent delusions), disorganized speech and disordered thought pattern, as well as general bizarre and/or catatonic behaviors (Andreasen, 1995; Basso et al., 1998; Lewis & Lieberman, 2000). Negative symptoms are behaviors and subjective experiences that include alogia (lack of conceptual fluency and reduced speech output), affective blunting (lack of emotional expression), anhedonia (inability to experience pleasure), avolition (lack of motivation), as well as cognitive deficits that include impairments in sustained and span of attention, working/reference memory, and executive functions (Andreasen, 1995; Basso et al., 1998; Lewis & Lieberman, 2000; Rowley et al., 2001). While each of these symptoms are present in some patients, each patient with schizophrenia has a different subset and degree of symptoms (Andreasen, 1995).

Cognitive impairments are clinically classified as a negative symptom, but are a core feature of schizophrenia that varies in degree of severity between affected individuals. Cognitive impairments can affect working memory, reference memory, attention, and executive functioning (Bora et al., 2010; Green et al., 2004; Keefe & Fenton, 2007). Impairments involving cognitive function can have profound effects on an individual's quality of life, compromising their ability to maintain relationships, employment, or even appropriate daily hygiene practices (Ho et al., 2000; Ho et al., 1998; Silver et al., 2003). Additionally, primary symptoms of schizophrenia can facilitate secondary symptoms, such as depression (Glazer et al., 1981; Prusoff et al., 1979; Siris, 1991). Afflicted individuals constitute 1/3 of the homeless population, and suicide rates are very high at roughly 10% (Inskip et al., 1998; Palmer et al., 2005; Regier et al., 1993; Rowley et al., 2001).

Schizophrenia affects approximately 24 million (1.1%) people worldwide (Saha et al., 2005), and has an early age of onset that varies between gender and individual. Schizophrenia is a disease that affects young people and has a typical on-set in either their late teens or early twenties (Andreasen, 1995). The early onset contributes to schizophrenia being one of the most costly diseases since adequate treatment is necessary for the remainder of the person's life (Andreasen, 1995; Messias et al., 2007). Total costs include drug treatment, residential accommodation, physician, and other healthcare services, which are estimated at $11.1 billion (Andreasen, 1995). In terms of lost productivity, additional costs to society are estimated at $20 billion (Andreasen, 1995; Knapp, 1997; Rowley et al., 2001).

The etiology of schizophrenia remains very difficult to determine, as ethnicity, geographic region, season of birth, and socioeconomic environment have not been useful predictors for this disease (Messias et al., 2007). However, there is a strong link between genetic factors and the development of schizophrenia. Studies involving monozygotic twins report a 70-80% concordance with schizophrenia (Cardno et al., 1999; Lichtenstein et al., 2009; Uher, 2014). These findings suggest that there is a genetic risk for schizophrenia, but that non-genetic factors, such as the environment and/or stress, play a role as well.

Hypotheses for the Etiology of Schizophrenia

Neurodevelopemental Hypothesis

The neurodevelopmental hypothesis suggests that the pathophysiology of schizophrenia involves genetic factors, which begin before the brain approaches maturity. Subtle molecular abnormalities coupled with stressful environmental factors during critical developmental periods can lead to inefficient signaling in brain circuitries, which may cause the symptoms of schizophrenia (Brown et al., 2004; Fatemi et al., 2005; Krapelin, 1893). Keshavan's "two-hit" model suggests that when insults occur during critical developmental periods, they can ultimately lead to brain maldevelopment and ultimately development of the disease. Critical developmental periods include early brain development, possibly during the second trimester and adolescence. During early brain development abnormal neuronal migration or connectivity may occur, and during adolescence excessive synaptic pruning may occur leading to loss of plasticity and proper

neural connectivity (Keshavan, 1999; Keshavan et al., 1999). Environmental factors linked to a higher concordance of schizophrenia may include prenatal viral or bacterial infections, obstetric complications, and other postnatal external stressors (Karlsson et al., 2001; Lewis, 2001; Schmidt-Kastner et al., 2006).

Magnetic resonance imaging (MRI) studies and post-mortem analyses support the neurodevelopmental hypothesis, reporting significant anatomical brain differences between schizophrenia patients and age-matched control subjects. Physiological differences that are present at the on-set of schizophrenia symptoms and progress slowly include: enlarged ventricles, decreased prefrontal cortical volume, decreased amygdala and hippocampal volumes, as well as deficits in gray matter volume in the left superior temporal gyrus, insular cortex, left medial temporal lobe, and ares of the prefrontal cortex (PFC) including the anterior cingulate and medial frontal gyri (Breier et al., 1992; Petronis et al., 1999; Sigmundsson et al., 2001).

Given the executive control functions of the PFC over many aspects of cognition, the overall reduced cortical volume and activity, as well as retracted dendrites and dendritic spines observed in the PFC may be involved in the cognitive impairments associated with schizophrenia (Glantz & Lewis, 2000; Petronis et al., 1999). Dendritic spine density and morphology change due to processes such as learning and memory, and these changes are critical for maintaining proper synaptic functioning. Imaging studies in the rodent neocortex show that small spines predominantly contain N-methyl-D-aspartate (NMDA) type glutamate receptors and are highly plastic. In contrast, large spines predominantly contain α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type glutamate receptors, and are highly stable (Bourne & Harris, 2007; Holtmaat et al.,

2006; Kasai, 2006; Matsuzaki et al., 2001; Zui et al., 2005). These observed differences have led to suggestions that small spines are required for learning new information, and stable spines may be required for proper functioning of circuits that underlie memory (Bourne & Harris, 2007; Kasai et al., 2003; Kasai et al., 2010). Thus, the loss of dendritic spine density and atrophy of existing dendritic spines in patients with schizophrenia may underlie impaired stability of synaptic function and lead to the observed cognitive impairments.

Underlying processes for the neurodevelopmental hypothesis involve genes that may be involved in the risk of developing schizophrenia, known as susceptibility genes (Moises et al., 1995). The most prominent susceptibility genes include dysbindin (DTNBP1), neuregulin (NRG1) and disrupted in schizophrenia 1 and 2 (DISC1 and DISC2) (Le-Niculescu et al., 2007; Sullivan et al., 2006; Owen et al., 2005). These genes encode proteins that aid in regulating neuronal connectivity and synaptogenesis (Fatemi & Folsom, 2009). DISC1 may be partly responsible for the physical brain abnormalities, as its gene product modulates downstream proteins, which are responsible for neural migration and neural progenitor proliferation during embryonic brain development and in the adult hippocampus (Mao et al., 2009). Mutations in these genes are likely involved in the maldeveloped formation of specific neuronal pathways, involving glutamate and dopamine (DA), both of which have been suggested to be involved in schizophrenia-like symptoms (Stefansson et al., 2002; Stefansson et al., 2003; Liu et al., 2007).

Glutamate Hypothesis

A second hypothesis for the etiology of schizophrenia evolved from case studies of individuals who exhibited psychotic symptoms similar to schizophrenia while under the influence of drugs such as phencyclidine (PCP) or ketamine (Allen & Young, 1978; Cohen et al., 1962; Javitt & Zukin, 1991; Krystal et al., 1994). PCP and drugs with a similar profile noncompetitively block N-methyl-D-aspartate (NMDA) type glutamate receptors and induce both positive and negative symptoms of schizophrenia, as well as cognitive impairments, resulting in a drug state that closely resembles schizophrenia. Specifically, the glutamate hypothesis proposes that the pathology of schizophrenia is due to hypofunctioning NMDA receptors (NMDAR) (Marek et al., 2010; Olney et al., 1999; Paz et al., 2008; Tsai & Coyle, 2002). This hypothesis suggests that hypofunctional NMDAR, which are located on glutamatergic neurons and synapse onto DA neurons in the ventral tegmental area (VTA), are unable to excite those DA neurons. The lack of DA excitation may result in DA hypofrontality, and thus cause symptoms of cognitive impairments (Marek et al., 2010; Olney et al., 1999).

The competing hyperglutamatergic "excitotoxicity" hypothesis (Gray & Roth, 2007; Javitt, 2004) is based on the observation that NMDAR antagonists cause neurodegeneration of pyramidal neurons following acute and/or chronic administration (Gray & Roth, 2007; Javitt, 2004; Olney & Farber, 1995; Olney et al., 1999). This model suggests that the symptoms of schizophrenia may be due to NMDAR antagonist induced apoptotic changes, particularly in frontocingulate regions (Gray & Roth, 2007; Javitt, 2004; Olney & Farber, 1995; Olney et al., 1999). Furthermore, hypofunctioning NMDAR may be due to compensatory mechanisms in response to previous chronic hyper-

functioning glutamate signaling casuing excitotoxic neuronal cell death (Deutsch et al., 2001; Javitt, 2004). In support of this hypothesis, NMDAR antagonists have been shown to increase extracellular glutamate levels in the mPFC and nucleus accumbens (NAC) (Adams & Moghaddam, 2001; Lena et al., 2006; Lopez-Gil et al., 2007, Lopez-Gil et al., 2009; Lorrain et al., 2003; Moghaddam et al., 1997; Pietraszek et al., 2009; Roenker et al., 2011; Roenker et al., 2012; Zuo et al., 2006); and treatment naïve patients with schizophrenia were found to have significantly higher levels of glutamate in their cerebrospinal fluid (CSF) (Hashimoto et al., 2005). Lastly, memantine, a weak NMDAR antagonist, was shown to slow cognitive decline in advanced Alzheimer's disease (Reisberg et al., 2003). This result suggests that excessive glutamate transmission contributes to neurodegeneration and cognitive decline in Alzhiemer's disease, and thus possibly in schizophrenia as well.

Dopamine Hypothesis

The DA hypothesis suggests that hyperdopaminergic neurotransmission in the VTA results in the positive symptoms of schizophrenia. In a review of the dopamine hypothesis, Meltzer and Stahl (1976) suggest that schizophrenia symptoms are caused by excessive DA in the brain, particularly in the mesocorticolimbic DA pathway. This pathway begins in the VTA and projects to various regions in the limbic system and the prefrontal cortex (Meltzer & Stahl, 1976).

The DA hypothesis originated from studies showing that antipsychotic drugs exert their effects by antagonizing D_2-like receptors. Researchers provide evidence for this hypothesis by showing that antipsychotics inhibit the release of dopamine in rat striatal

slices (Seeman & Lee, 1975). This hypothesis gained support from many other studies that reported acute psychosis after amphetamine overdose, and produced behaviors indistinguishable from paranoid schizophrenia. Behaviors include disorder of thought, paranoid delusions, and auditory hallucinations (Beamish & Kiloh, 1960; Greenwood & Peachey, 1957; McConnell & Mcllwaine, 1961; O'Flanagan & Taylor, 1950). Amphetamine induces these symptoms by increasing the release of monoamines, and thus an overabundance of DA in the synapse.

Revisions to the DA hypothesis add the possibility of an inverse relationship between excessive DA in the limbic system and diminished DA in the PFC. Therefore, revisions to the DA hypothesis offer a possible explanation for all primary symptoms associated with schizophrenia, rather than just the positive symptoms (Davis et al., 1991; Howes & Kapur, 2009). Decreased activity in the mesocortical DA pathway may lead to decreased DA in the PFC, which may underlie the cognitive and negative symptoms observed in schizophrenia patients (Brozoski et al., 1979; Davis et al., 1991; Goldman-Rakic et al., 2004).Thus, it is suggested that there is an opposing relationship between these DA pathways, which include hyperdopaminergic activity in the mesolimbic pathway, and hypodopaminergic activity in the mesocortical pathway.

Mesocorticolimbic Dopamine System

The mesolimibic and mesocoritcal DA pathways are critical for explaining the suggested physiology that may underlie the symptoms of schizophrenia. The glutamate and DA hypotheses for the etiology of schizophrenia are focused on dysfunctional neuronal signaling occurring within these pathways. The major components in the

mesocorticolimbic circuit are the PFC, amygdala, NAC, striatum and the VTA. The NAC is divided into the shell and the core. The shell sends efferent projections to several regions, including but not limited to, the ventromedial ventral pallidum, amygdala, preoptic area, hypothalamus, endopeduncular nucleus, and VTA (Ikemoto & Panksepp, 1999). The core sends major efferent projections to several regions as well, which include the dorsolateral ventral pallidum, endopeduncular nucleus, lateral part of the VTA, and substantia nigra. The VTA receives afferent inputs from the PFC, NAC, BNST, lateral preoptic area, and lateral hypothalamus from frontal regions, and the superior colliculus, substantia nigra, dorsal raphé, parabrachial nucleus, and dentate nucleus of the cerebellum from brainstem regions. The NAC receives afferent inputs from frontal regions, which include the mPFC, amygdala, hippocampus, and thalamus, but also from the VTA, dorsal raphé, and mesopontine reticular formation (Ikemoto & Panksepp, 1999). The PFC sends excitatory projections to the VTA that stimulate GABAergic interneurons and mesocortical dopamine neurons (Carr & Sesack, 2000). Infralimbic (IL) and prelimbic (PL) both project to the VTA (Vertes, 2004). PL projections extend to the NAC core and shell, but IL projections extend to the NAC shell (Vertes, 2004). The basolateral amygdala distributes excitatory projections to DA neurons in the midbrain and NAC (Cooper, 2002).

Dysfunctional excitatory projections in the mesocorticolimibic DA system may be responsible for the abnormal DA signaling that is suggested to explain the symptoms of schizophrenia. Thus, the mesocorticolimbic DA system provides a physical link between the glutamate hypothesis of schizophrenia and the DA hypotheses for schizophrenia. Stimulation of VTA DA neurons from PFC excitatory efferent projections is suggested to

be disrupted in patients with schizophrenia. Hypofunctioning NMDAR on VTA DA neurons cause decreased DA release in the mesocortical DA pathway, resulting in too little DA in the frontal cortex (Olney & Farber, 1995; Olney et al., 1999). At the same time, Hypofunctioning NMDAR on GABAergic interneurons are unable to inhibit DA release in the mesolimbic DA pathway and result in too much DA, thus providing an explanation for the suggested inverse relationship of the DA hypothesis of schizophrenia (Olney & Farber, 1995; Olney et al., 1999).

Animal Models of Schizophrenia

Animal models of schizophrenia are essential for advancements in the production and screening of novel therapeutic compounds. However, developing a model that has construct, face, and predictive validity for a disease as complex as schizophrenia has proven difficult. Current models of schizophrenia can be classified into four main groups: pharmacological administration, developmental manipulation, genetic manipulation, and lesioning models. Each of these models has different strengths and weaknesses, and currently no animal model is able to adequately express all symptoms of schizophrenia. An appropriate model of schizophrenia should include face validity, expressing observable symptoms of schizophrenia. In animal models, positive symptoms are observed as hyperlocomotion and sensitization to stimulant effects, such as amphetamine. Negative symptoms are observed as social withdrawal and increased aggression. Cognitive impairments are observed as deficits in sensorimotor-gating (pre-pulse inhibition, PPI), as well as attention, working memory, and reference memory tests. Animal models should also include construct validity, which involves replicating the

theoretical pathophysiology of schizophrenia. Lastly, an adequate animal should also have predictive validity, which is demonstrated by the expected response to treatment by current antipsychotics (Jones, Watson, & Fone, 2011).

Pharmacological Models of Schizophrenia

The amphetamine model of schizophrenia is based on the DA hypothesis, and thus targets hyperactivity in the mesolimbic DA pathway. Amphetamine-induced psychosis produces positive symptoms that are very similar to schizophrenia, which include auditory hallucinations and persecutory delusions (Robinson & Becker, 1986). Chronic amphetamine administration in animals causes a robust model of the positive symptoms of schizophrenia, which are observed as hyperlocomotor activity and amphetamine sensitization (Featherstone et al., 2008; Robinson & Becker, 1986). However, neither acute nor chronic administration of amphetamine is able to induce the negative symptoms of schizophrenia (Featherstone et al., 2007; Javitt & Zukin, 1991). Amphetamine administration has been shown to cause long-lasting PPI deficits (Tenn et al., 2005; Peleg-Raibstein et al., 2006). Additonally, amphetamine sensitization was shown to impair attention and set-shifting ability, but not performance in spatial tasks (Featherstone et al., 2007; Fletcher et al., 2005). Pre-administration of clozapine or haloperidol demonstrated the ability to prevent amphetamine-induced sensitization (Martinez & Sarter, 2008; Meng et al., 1998). Thus, the amphetamine model induces psychotic like symptoms, but it is unable to replicate negative symptoms, or most cognitive impairments that are associated with schizophrenia.

The phencyclidine (PCP) model of schizophrenia is based on the glutamate hypothesis of schizophrenia, and was formed by observing people under the influence of NMDA antagonists (i.e., PCP, ketamine or MK-801). These observations led to the suggestion that dysfunctional glutamate circuitries may be the primary mechanism mediating the pathophysiology of schizophrenia (Coyle et al., 2003; Konradi & Heckers, 2003; Olney & Farber, 1995; Tsai & Coyle, 2002). In healthy subjects, administration of PCP induces psychosis, as indicated by delusions and hallucinations that are similarly observed as positive symptoms of schizophrenia (Cohen et al., 1962; Krystal et al., 1994). In schizophrenia patients, PCP can cause the onset or perpetuation of psychosis (Javitt & Zukin, 1991). Also in humans, acute and chronic PCP administration induces negative symptoms, such as social withdrawal and poverty of speech (Luby et al., 1959; Sams-Dodd, 1996), as well as cognitive impairments. PCP induced symptoms are reversible with cessation of drug use (Cosgrove & Newell, 1991; Javitt & Zukin, 1991). In rodents, PCP administration causes hyperlocomotion (Kalinichev et al., 2008) social withdrawal (Sams-Dodd, 1995), PPI deficits (Mansbach & Geyer, 1989), as well as impairments in learning, attention, working memory and reference memory (Egerton et al., 2005; Jentsch et al., 1997; Marquis et al., 2007; Seillier & Giuffrida, 2009). The observed positive and negative symptoms are reversed by administration of both typical and atypical antipsychotics (Phillips et al., 2001; Sams-Dodd, 1998), but cognitive impairments are reversed only by atypical antipsychotics (Le Cozannet et al., 2010; Goetghebeur & Dias, 2009). Chronic PCP administration results in neurochemical changes that are similar to those believed to underlie the pathophysiology of schizophrenia. This is supported by chronic PCP administration, which demonstrates that mesolimbic DA neurons are hyper-

responsive to amphetamines and mild stress (Jentsch et al., 1998). Furthermore, chronic PCP administration results in reduced basal glutamate release in the PFC (Fattorini et al., 2008), and increases PFC levels of the glutamate-aspartate transporter (GLAST), which suggests a mechanism for cortical glutamate hypofunction (Murai et al., 2007). Sub-chronic PCP administration decreases dendritic spine density on cortical neurons (Flores et al., 2007), and reduces numbers of both cortical and hippocampal parvalbumin-immunoreactive neurons (Abdul-Monim et al., 2007; Jenkins et al., 2008; Jenkins et al., 2010; McKibben et al., 2010; Reynolds et al., 2004), which are also reflective of the pathophysiology observed in schizophrenia patients. Overall, NMDAR antagonists display high face, predictive and construct validity, and thus adequately model schizophrenia symptoms in laboratory animals.

Although MK-801 and PCP share the same mechanism of action, and produce substantial overlap on behavioral effects, there are significant differences between the two. On the side of similarities, both drugs are NMDAR antagonists and have the same binding site on the receptor (Hirmatsu, Cho, & Nabeshima, 1989). Both stimulate ataxia, sniffing, locomotion, headweaving, turning, and backpedaling (Hirmatsu et al., 1989). Both are used as models of schizophrenia (Jones et al., 2011), and both stimulate DA turnover by releasing stored DA in vesicles, rather than releasing newly synthesized DA (Martin & Haubrick, 1985). Differences between MK-801 and PCP include binding specificity and potentcy. MK-801 has produced no evidence of effects on other receptor systems, with the exception of mediating the release of stored DA, which PCP also iniates (Nabeshima et al., 1983; Nabeshima et al., 1984). Alternatively, PCP has been shown to bind to other receptors, such as the sigma receptor, and is also potent 5-HT re-

uptake inhibitor (Hirsch et al., 1997; Nabeshima et al., 1983; Nabeshima et al., 1984). MK-801 is indicated to be 20-40 times more potent than PCP (Hirmatsu et al., 1989). MK-801 induces dose-dependent behavioral effects (hyperactivity, ataxia, sniffing, ect.) at significantly lower doses than PCP (Hirmatsu et al., 1989). Also of notable importance, MK-801 has a much slower time course to reach maximum behavioral effects than PCP. MK-801 maximum effects occur between 30-45 minutes and PCP maximum effects occur between 0-15 mins (Himatsu et al., 1987; Nabeshima et al., 1986; Wong et al., 1986).

Neurodevelopmental Disruption Models of Schizophrenia

Neurodevelopmental models of schizophrenia are based on the neurodevelopmental hypothesis. This hypothesis suggests that a genetic predisposition and an early-life stressor can trigger maldevelopment of the brain, and possibly the onset of schizophrenia symptoms. Thus, developmental models utilize environmental manipulations and/or drug administration during critical developmental periods to cause changes in CNS development (Jones et al., 2011).

In one model, administration of the neurotoxin methylazoxymethanol acetate (MAM) administration on gestational day 17 (GD17) has been shown to cause hyperlocomotion, (Lodge & Grace, 2007), PPI deficits (Talamini et al., 2000), and impairments in reversal-learning and set-shifting tasks (Gourevitch et al., 2004; Le Pen et al., 2006; Moore et al., 2006). Only one study evaluated the effect of antipsychotics on MAM-induced impairments. This study showed that neither clozapine nor haloperidol were able to reverse any of the above mentioned impairments (Fiore et al., 2007). Subtle

physiological abnormalities that are similar to those observed in patients with schizophrenia have been observed in this model. These abnormalities include reductions in cortical thickness throughout the mPFC, hippocampus, and parahippocampal cortices, and disorientation of pyramidal neurons in the hippocampus (Flagstad et al., 2004; Kovelman et al., 1984; Le Pen et al., 2006; Moore et al., 2006).

The post-weaning social isolation model utilizes social deprivation to alter brain development, and cause behavioral deficits that are evident in adulthood (Fone & Porkess, 2008; Lapiz et al., 2003). Rodents undergoing post-weaning isolation show locomotor hyperactivity (Dalley et al., 2002; Del Arco et al., 2004; Fone et al., 1996; Silva-Gomez et al., 2003), deficits in PPI (Bakshi et al., 1998; Cilia et al., 2001; Weiss et al., 1999) and impaired working memory, reference memory and cognitive flexibility (Dalley et al., 2002). Antipsychotics either fully or partially reverse the impairments that are observed as either positive or negative symptoms (Bakshi et al., 1998; Cilia et al., 2001; Varty & Higgins, 1995; Wilkinson et al., 1994). Cognitive impairment reversal has been minimally examined, but chronic clozapine administration resulted in reduced impairment in a reversal learning task (Li et al., 2007). Neurochemical and structural changes observed in this model include increased DA in the mesolimbic pathway (Jones et al., 1992; Hall et al., 1998; Fone & Porkess, 2008), reduction in frontal cortical matter (Day-Wilson et al., 2006; Schubert et al., 2009), and reduced dendritic spine density and spine morphology (Silva-Gomez et al., 2003; Pascual & Zamora-Leon, 2006).

Genetic Models of Schizophrenia

Genetic models include knock-out (KO) or knock-down (KD) transgenic rodent strains in an effort to produce changes in mRNA and proteins in order to develop models of schizophrenia endophenotypes which may manifest underlying processes for specific susceptibility, or risk genes (Braff et al., 2007; Gottesman & Gould, 2003; O'Tuathaigh & Waddington, 2010). Of the many risk genes implicated in schizophrenia, some of the most prominent are DISC1, neuregulin and dysbindin.

DISC1 has long been thought to be involved in the pathophysiology of schizophrenia. DISC1 is a synaptic protein expressed early in development and exerts a prominent role in pre- and post-natal neuronal development. DISC1 is particularly involved in synaptogenesis, neuronal migration and synaptic plasticity (Jaaro-Peled, 2009), all of which are crucial for the proper physiological CNS structure. Some DISC1 transgenic mice studies have reported hyperlocomotor activity (Clapcote et al., 2007), while other studies did not observe this (Koike et al., 2006; Li et al., 2007; Pletnikov et al., 2008). Social withdrawal was evident in some studies (Clapcote et al., 2007; Desbonnet et al., 2009; Li et al., 2007) whereas other studies did not observe this behavior (Hikida et al., 2007). Cognitive impairments observed in DISC1 mutant mice include deficits in PPI (Clapcote et al., 2007; Hikida et al., 2007), and working memory and executive function (Pletnikov et al., 2008). However, spatial tasks and novel object recognition are not affected (Arguello & Gogos, 2010; Hikida et al., 2007; Kvajo et al., 2008). PPI deficits were reversed by haloperidol and clozapine (Clapcote et al., 2007; Hikida et al., 2007). Unfortunately no studies have examined the reversal of positive symptoms or cognitive impairments in DISC1 mice by administration of antipsychotics (Jones et al., 2011). DISC1 transgenic mice show physiological changes similar to those

observed in schizophrenia including enlarged lateral ventricles and reduced cortical thickness (Jaaro-Peled et al., 2010). In some but not all studies, DISC1 mutant mice demonstrate reduced parvalbumin immunoreactivity in the mPFC and hippocampus, which may underlie cognitive impairments (Clapcote et al., 2007; Hikida et al., 2007; Shen et al., 2008; Jaaro-Peled et al., 2010). Also, abnormal dendritic structure and density in the hippocampus have been observed in some DISC1 transgenic mice (Li et al., Li et al., 2007b; Kvajo et al., 2008).

Neuregulin (NRG1) is involved in several crucial development functions such as synaptogenesis, neuronal migration, myelination, neuron glial interactions and formation of glial cells (Harrison & Law, 2006; Mei & Xiong, 2008; van den Buuse et al., 2009). Homozygous KO of NRG1 is fatal, however several viable heterozygous or hypomorphic/conditional KO mice have been developed and display schizophrenia-like symptoms (Harrison & Law, 2006a; Mei & Xiong, 2008). Between the many NRG1 heterozygous or hypomorphic/conditional KO mice strains, there is a lot of symptom variability, but in general, NRG1 transgenic mice display hyperlocomotor activity in open-field and Y-maze tasks (O'Tuathaigh et al., 2007; Stefansson et al., 2002; van den Buuse et al., 2009), and abnormal socialization involving increased aggression and in some types social withdrawal (O'Tuathaigh et al., 2007; O'Tuathaigh et al., 2010). Cognitive impairments include PPI deficits (Stefansson et al., 2002), and impaired contextual fear conditioning (Arguello & Gogos, 2010; Ehrlichman et al., 2009), but working memory remained unaffected (O'Tuathaigh et al., 2007; O'tuathaigh et al., 2010). Administration of clozapine was shown to reverse hyperlocomotion (O'Tuathaigh et al., 2007; Stefansson et al., 2002; van den Buuse et al., 2009). NRG1 transgenic mice

have increased parvalbumin-positive neurons in the mPFC and reduced levels of DA in the mPFC and hippocampus, none of which is similar to the suggested pathophysiology of schizophrenia (Kato et al., 2010). Overall, there is a profound lack of consistency of schizophrenia-like behaviors in NRG1 transgenic mice, and paired with a general lack of pharmacological studies, this model has very little face, predictive or construct validity.

Dysbindin is a synaptic protein thought to regulate exocytosis, vesicle biogenesis and receptor trafficking in relation to excitatory neurotransmission (Karlsgodt et al., 2011; Papaleo et al., 2010). Dysbindin mutations have high correlations with the risk and on-set of schizophrenia in humans, making dysbindin one of the most promising candidate risk genes (Allen et al., 2008; van den Buuse et al., 2009; Williams et al., 2005). A naturally occurring mutation in mice [(named 'sandy' (sdy)] carries a homozygous, spontaneous deletion of exon 2 of the dysbindin gene, resulting in a naturally occurring loss of dysbindin (Papaleo et al., 2010). These dysbindin-lacking mice show hyperlocomotion (Cox et al., 2009; Papaleo et al., 2010; van den Buuse, 2010) and hypersensitivity to amphetamine-induced locomotion (Papaleo et al., 2010). Social withdrawal was reported in these mice (Papaleo et al., 2010), and cognitive impairments that have been observed include abnormal PPI responses (Feng et al., 2008; O'Tuathaigh et al., 2010), deficits in spatial reference memory and novel object recognition, but enhanced fear conditioning (Arguello & Gogos, 2010; Bhardwaj et al., 2009; Feng et al., 2008; Takao et al., 2008). The increased PPI observed in these mice has been shown to be reversed by administration of quinpirole (Papaleo et al., 2010). Physiological alterations in dysbindin mutant mice include abnormal dendritic spine morphology in excitatory synapses in the hippocampal CA1 region, and reduced number of presynaptic

glutamate-containing vesicles (Chen et al., 2008; Feng et al., 2008; Jaaro-Peled et al., 2010).

Lesion Models of Schizophrenia

In lesion models of schizophrenia, site-specific lesions produced on postnatal day 7 by local injection of ibotenic acid into the ventral hippocampus (Becker et al., 1999; Lipska et al., 1993) cause behaviors similar to schizophrenia which do not appear until after puberty (Tseng et al., 2009). This model produces hyperlocomotor activity (Lipska et al., 1993) and hypersensitivity to DA agonists (Lipska & Weinberger, 1993). Deficits include social withdrawal and aggression (Sams-Dodd et al., 1997). Cognitive impairments include deficits in PPI (Le Pen et al., 2000), impaired spatial learning, and impaired working memory (Chambers et al., 1996). Both haloperidol (Sams-dodd et al., 1997) and risperidone (Richtand et al., 2006) have been shown to reduce amphetamine induced hyperlocomotor activity in this model. However, clozapine was unable to reverse neither social withdrawal nor aggression (Sams-Dodd et al., 1997). In vivo microdialysis studies have shown DA levels to be unaltered in the nucleus accumbens in this model (Brake et al., 1999; Wan & Corbett, 1997), but reductions in dendritic spine density and dendritic length of pyramidal neurons in the mPFC and nucleus accumbens were observed (Flores et al., 2005; Marquis et al., 2008

Antipsychotic Drugs

Typical or First Generation Antipsychotics

Chlorpromazine was developed in 1950 as a pre-anesthetic drug, but it was observed to have a profound calming effect when administered to psychiatric patients. Chlorpromazine became the first line of treatment for psychosis (Meyer & Simpson, 1997). In the following years several other drugs similar to chlorpromazine were developed, and these drugs became known as "typical" antipsychotics (APDs). Typical APDs reduce the positive symptoms of schizophrenia by acting as D2-like receptor antagonists and thus counteract the hypothesized hyperactive mesolimbic DA pathway. However, this same mechanism of action causes adverse side effects such as extrapyramidal side effects (EPS). EPS are a cluster of side effects that are Parkinsonian-like state characterized by tremors, muscle rigidity, dystonia, and dyskinesia (Haro & Salvadr-Carulla, 2006; Owens, 1999). EPS is specifically caused by D_2-like receptor inhibition in the nigrostriatal DA pathway which reduces DA transmission in the basal ganglia, causing motor disruption (Haro & Salvadr-Carulla, 2006; Owens, 1999). Also, D_2-like receptor inhibition in the tuberoinfundibular DA pathway causes increased release of the hormone prolactin. Prolactin regulates reproductive functions, and an imbalance of this hormone can result in galactorrhea, amenorrhea and other possible sexual dysfunctions (Arana, 2000; Marken et al., 1992).

Atypical or Second Generation Antipsychotics

Clozapine was the first atypical or second generation antipsychotic to be developed (Hippius, 1999). Clozapine was considered atypical because it did not produce

the expected side effects of typical antipsychotics such as EPS, prolactin elevation or catalepsy, which during that time were thought to be general properties of antipsychotic drugs. Atypical APDs are termed "atypical" due to the absence of the typical side effects, and have a different pharmacological profile. Atypical APDs target several binding sites, but have the highest affinity for the serotonin 5-HT2A receptor and relatively weaker affinity for dopamine D2-like receptors (Meltzer, Matsubara, & Lee, 1989). Atypical APDs lessen the risk for EPS and other side effects through the addition of 5-HT_{2A} receptor antagonism and/or 5-HT_{1A} agonism to their binding profile. Specifically, 5-HT_{2A} antagonism and 5-HT_{1A} agonism actually increase DA release just enough to reduce EPS, excessive prolactin release, and still alleviate positive symptoms (Kim et al., 2009; Stahl, 2003). Atypical APDs may also lessen the risk of typical APD side effects by dissociating more quickly from receptors. This so-called 'fast-off-D_2' or 'rapid dissociation' is based on the lower occupancy and affinity of atypical APDs for D2 receptors, thereby lessening DA transmission enough to reduce positive symptoms, while maintaining enough DA transmission to avoid related side effects (Seeman, 2002; Seeman, 2005; Stahl, 2003).

Atypical APDs have complex mechanisms of action that have different affinities for different receptors that lead to varying effects on schizophrenia symptoms as well as varying side effects (Meyer & Simpson, 1997). Aside from targeting D_2 and 5-HT_{2A} receptors, atypical APDs also have effects on adrenergic, cholinergic, and histaminergic receptors (Schotte et al., 1996). The effects of targeting the aforementioned receptors cause adverse side effects including sedation, weight gain, dizziness and constipation among others (Fleischhaker et al., 2006; Hori et al., 2006; Lavalaye et al., 2001;

Lieberman, 2004; Raedler, 2007). Commonly prescribed atypical APDs include risperidone, olanzapine, ziprasidone, and quetiapine (Roth, Sheffler, & Kroeze, 2004).

Third Generation Antipsychotics

A third class, or generation, of APDs has been recently developed. The prototype drug for this class is aripiprazole and has a different mechanistic profile than other atypical APDs. Aripiprazole and similar drugs such as bifeprunox have a greater affinity for DA receptors than other atypical antipsychotics, and instead of inhibiting D_2-like receptors, these novel treatments act as partial agonists at D_2-like receptors (Burris et al., 2002). Because of this unique profile, both aripiprazole and bifeprunox have a lower propensity to elicit EPS despite their high affinity for DA receptors (Newman-Tancredi et al., 2007). Aripiprazole and bifebrunox bind to both D_2 and D_3 as well as 5-HT_{1A} receptors (Ohlsen et al., 2005; Tadori et al., 2007; Tadori et al., 2008), and act to stabilize DA levels in the synapse, depending on the electrophysiological and biochemical state of the cell (Burris et al., 2002).

Despite these pharmacological advancements, there is conflicting evidence for the efficacy of atypical APDs over typical APDs. Although several studies report that atypical APDs significantly benefit negative and cognitive impairments, other studies report either no improvements, or slight improvements, in negative and cognitive function over typical APDs (Geddes et al., 2000; Woodward et al., 2005). Meltzer and McGurk (1999) assessed how atypical antipsychotic drugs, clozapine, risperidone, and olanzapine affected various cognitive functions. Although it was found that the atypical APDs were able to improve some aspects of cognition, none showed improvements for

all cognitive domains. Risperidone improved working memory, executive function and attention (Green et al., 1997). Clozapine improved attention and verbal fluency, whereas olanzapine improved verbal learning and memory (Meltzer and McGurk, 1999; Silver et al., 2003). However, there have been discrepancies regarding the notion that atypical APDs benefit the negative and cognitive symptoms more so than typical APDs. Large scale studies such as the Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) and Cost Utility of the Latest Antipsychotic drugs (CUtLASS), have reported that atypical APDs were not necessarily superior over typical APDs. The most interesting result from both studies was the lack of significant differences found between typical APDs and atypical APDs efficacy for treating the negative symptoms and cognitive impairments (Jones et al., 2006; Lewis & Lieberman, 2008; Stroup et al., 2003). Thus, addition research and drug development are necessary to further improve the efficacy of current treatments for schizophrenia.

The Glutamate System

Glutamate is the most prevalent excitatory neurotransmitter in the central nervous system, mediating as much as 70% of both fast and slow excitatory neurotransmission (Olive, 2009). Glutamate receptors are either ligand-gated ion channels (i.e., ionotropic glutamate receptors, or iGluRs) or G-protein coupled receptors (i.e., metabotropic glutamate receptors, or mGluRs). iGluRs are ion channels that are permeable to cations and function by allowing Na^+ and Ca^{2+} to enter the cell initiating action potentials and activating various intracellular signaling pathways. iGluRs are located on the head of postsynaptic dendritic spines, mediate fast excitatory neurotransmission, and are divided

into three different subtypes: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), and kainic acid (KA) (Olive, 2010). mGluRs are located on both presynaptic terminals and postsynaptic dendritic spines (Conn & Pin, 1997; Hermans & Challiss, 2001; Pin & Duvoisin, 1995). mGluRs mediate slower, modulatory neurotransmission, and based on their intracellular signaling and pharmacological properties, are categorized into three families. Group I mGluRs consists of mGluR1 and mGluR5 receptors, Group II mGluRs consists of mGluR2 and mGluR3 receptors, and Group III consists of mGluR4, mGluR6, mGluR7 and mGluR8 receptors (Olive, 2009).

Group I mGluR Signaling Mechanisms

Group I mGluRs primarily utilize G_q/G_{11} signaling mechanisms that activate phosopholipase C to form diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG activates protein kinase C (PKC), while IP_3 exerts further downstream effects by binding to IP_3 receptors on the endoplasmic reticulum releasing intracellular Ca^{2+} which in turn activates other intracellular messengers such as calcium/calmodulin-dependent kinase II (CaMKII). PKC exerts further downstream effects by phosphorylating CREB and other signaling molecules, which can eventually lead to altered gene transcription (Hermans & Challiss, 2001). Other intracellular signaling targets of Group I mGluRs include pathways involving $G_{i/o}$ and G_s signaling, as well as the mitogen-activated protein kinase/extracellular signal-related kinase (MAPK/ERK) and mammalian target of rapamycin (mTOR)/p70 S6 kinase pathway, both of which are highly implicated in the

regulation of synaptic plasticity (Hou & Klann, 2004; Iacovelli et al., 2002; Li et al., 2007; Page et al., 2006; Saugstad & Ingram, 2008).

Group I mGluRs Link to NMDA Receptors

Group I mGluRs, particularly mGluR5, are physically linked to NMDA receptors by various scaffolding proteins (such as Homer, Shank and GKAP) and positively modulate NMDA receptor function. Through its activation of PKC, mGluR5 activation also increases the phosphorylation of NMDA receptor subunits, thereby indirectly increasing the probability of NMDA receptor channel opening (Niswender & Conn, 2010). Due to the postsynaptic localization of Group I mGluRs, these receptors primarily initiate and regulate long-term potentiation (LTP) and long-term depression (LTD) by postsynaptic mechanisms such as potentiation of NMDA receptor function (Anwyl, 1999; Bellone et al., 2008; Gladding et al., 2009). These structural and biochemical interactions allow for the stimulation of group I mGluRs to activate NMDA receptors with less risk of inducing excitotoxicity (Albensi, 2007; Hardingham & Bading, 2003).

Positive Allosteric Modulation of mGluR5 Receptors: Pro-cognitive Effects

Positive allosteric modulators (PAMs) are ligands that facilitate receptor functioning in the presence of glutamate binding to the orthosteric binding site. Thus, mGluR5 PAMs are more indirect routes for modulating NMDA receptor function that reduce the possibility of adverse side effects such as excitotoxicity. Several studies have examined the effects of the administration of mGluR5 PAMs on cognitive enhancing abilities in impaired and unimpaired cognitive states. For example, pharmacologically

induced cognitive deficits have been shown to be reversed by administration of mGluR5 PAMs in several different paradigms, including operant set-shifting tasks (Darrah et al., 2008; Gastambide et al., 2013; LaCrosse et al., 2014), spatial alteration tasks (Fowler et al., 2013; Stefani & Moghaddam, 2010), active allothetic place avoidance (Vales et al., 2010), reversal digging paradigms (Gastambide, 2012), social novelty discrimination (Clifton et al., 2013), and novel object recognition (Horio et al., 2013; Reichel et al., 2011). PAM of mGluR5 also reversed induced sucrose impairments (Vardigan et al., 2010) and taste aversion and inhibitory avoidance tasks (Fowler et al., 2011). Notably, mGluR5 PAMs have also been shown to improve spatial and recognition memory abilities in unimpaired animals (Ayala et al., 2009; Balschun et al., 2006; Uslaner et al., 2009; Xu et al., 2013). Operant set-shifting tasks are of particular importance for measuring cognitive functions that are PFC-dependent. Performance on operant based delayed match-to-sample (DMS) and delayed nonmatch-to-sample-tasks (DNMS) have been shown to be PFC-dependent tasks in several studies. Joel and colleagues (1997) found that mPFC lesions produced profound deficits in rodent performance on the DMS/DNMS task. Also, Sloan and colleagues (2006) showed that only rodents that received excitotoxic bilateral lesions to the mPFC, but not the hippocampus, exhibited significant impairments in delayed match-to-position (DMTP) water maze task. Rodents with hippocampal excitotoxic bilateral lesions performed no different than controls on this task, indicating that DMS/DNMS task are PFC-dependent tasks. Additionally, cognitive set-shifting such as DMS/DNMS is an executive function that involves attention, inhibition of response, working memory and planning/problem solving, all of

which have been associated with DLPFC (Duke & Kaszniak, 2000; Grafman & Litvan, 1999; Stuss & Alexander, 2000).

In contrast, various studies have shown that mGluR5 negative allosteric modulators (NAMs) impair cognitive function in otherwise unimpaired animals. For example, when administered systemically or via the intracerebroventricular route, mGluR5 NAMs can impair performance on spatial and working memory tasks (Balschun & Wetzel, 2002; Simonyi et al., 2010). Also, mGluR5 knockout (KO) mice show impaired performance in spatial memory tasks as well as acquisition and extinction of conditioned fear (Lu et al., 1997; Xu et al., 2009). However, not all studies have demonstrated cognitive deficits induced by mGluR5 NAMs (Petersen et al., 2002). mGluR5 NAMs have also been shown to improve aspects of cognitive impairment. Fragile X syndrome (FXS) is the most commonly inherited form of mental retardation (Gross et al., 2012). FXS is the result of either a deficiency or loss of function of the fragile X mental retardation 1 (FMR1) protein (McLennan et al., 2011). In FXS patients, mGluR5 NAMs such as 1-(3-chlorophenyl)-3-(3-methyl-5-oxo-4 H-imidazol-2-yl)urea (fenobam) have been shown to improve cognitive impairments (Berry-Kravis et al., 2009).

Reviewing the above listed published literature, mGluR5 PAMs have been shown to reverse induced deficits (Horio et al., 2013; LaCrosse et al., 2014; Reichel et al., 2011; Vales et al., 2010), and in some cases to enhance performance in learning and memory tasks in unimpaired animals (Ayala et al., 2009; Balschun et al., 2006; Uslaner et al., 2009; Xu et al., 2013). While in other cases mGluR5 PAMs have shown no evidence of cognitive performance enhancement, it is important to note that no evidence of

impairment or potentiation of impairment has been reported (Darrah et al., 2008; Stefani & Moghaddam, 2010). Alternatively, mGluR5 NAMs have been shown to cause performance deficits in cognitive based tasks in otherwise unimpaired rodents (Balschun & Wetzel, 2002; Simonyi et al., 2010).

It may be possible that the pro-cognitive effects of mGluR5 PAMs are due to enhanced dendritic spine plasticity. As discussed above, mGluR5 PAMs have been shown to be particularly successful in reversing cognitive impairments in animal models of schizophrenia such as PCP and MK-801(Darrah et al., 2008; LaCrosse et al., 2014; Stefani & Moghaddam, 2010). Patients with schizophrenia have been shown to have decreased dendritic spine density and atrophied spine size in the dorsolateral prefrontal cortex (DLPFC) and temporal regions (Glantz & Lewis, 2000; Hirsch et al., 1997), which have also been shown to have significantly less activity in schizophrenia patients during cognitive tasks as compared to control subjects (Tan et al., 2007). Acute and subchronic administration of MK-801 and other NMDAR antagonists in rodents at different developmental periods produce significant reductions in spine density in the mPFC, hippocampus, and striatum (Bellinger et al., 2002; Ramsey et al., 2010; Wedzony et al., 2005) Given that NMDAR antagonists model symptoms of schizophrenia, and also cause decreased dendritic spine density, this physiological effect may be an indicator of underlying mechanisms involved in impaired cognitive function in patients with schizophrenia. It is therefore possible that mGluR5 PAMs rescue observed behavioral cognitive deficits in models of schizophrenia by increasing dendritic spine plasticity.

3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB) is a PAM that acts on mGluR5, and may have potential as a cognitive enhancer that may be beneficial

for the treatment of cognitive deficits observed in schizophrenia patients. CDPPB binds within the seven transmembrane domain of the mGluR5 receptor and acts as a positive modulator that indirectly increases the receptor's activity. Group I mGluRs, which include mGluR1 and mGluR5 receptors, are densely expressed in areas of the brain necessary for cognition, such as the PFC, striatum and hippocampus (Stefani & Moghaddam, 2010).

Several studies have shown that CDPPB can rescue MK-801 induced cognitive deficits in behavioral tasks with efficacy similar to that of antipsychotics such as clozapine, which are known to have beneficial effects on the negative symptoms of schizophrenia. Stefani and Moghaddam (2010) found that CDPPB at doses of 10 mg/kg and 30 mg/kg were both able to attenuate MK-801 induced cognitive deficits in a T-maze set-shifting task. Vardigan and colleagues 2010 found that MK-801 decreased the rewarding properties of sucrose (demonstrated by a marked decrease in sucrose intake) but not water. Acute administration of CDPPB proved comparable to clozapine and D-serine in attenuating the MK-801 induced deficit (Vardigan et al., 2010). CDPPB has also shown to attenuate amphetamine induced hyper-locomotion and sensorimotor gating abnormalities, both of which lend support for further testing of this drug as a novel therapeutic target (Kinney et al., 2005). Together, these studies suggest that mGluR5 PAMs may be a promising new treatments for more complex cognitive deficits such as those associated with schizophrenia.

Dissertation Rationale

The purpose of this dissertation is to test the hypothesis that the mGluR5 PAM CDPPB will ameliorate induced cognitive impairments in an animal model of schizophrenia, increase structural plasticity of neuronal dendritic spines, and reverse pharmacologically increased extracellular levels of glutamate in the mPFC. Experiment 1 will assess the ability of CDPPB to reverse MK-801 induced perseverative responding in an operant set-shifting task employing delayed matching/non-matching-to-sample procedure. It is predicted that co-administration of CDPPB and MK-801 will decrease MK-801 induced perseverative responding, and that administration of CDPPB as a 30 min pre-treatment to MK-801 will prevent perseverative responding. Experiment 2 will test the effects of CDPPB on extracellular glutamate levels in the mPFC in naive and MK-801 treated rodents using *in vivo* microdialysis procedures. It is predicted that MK-801 administration will increase extracellular glutamate levels in the mPFC and that CDPPB will reverse that effect. Furthermore, it is predicted that administration of CDPPB alone will cause a decrease in levels of extracellular glutamate in the mPFC. Experiment 3 will measure the effects of CDPPB on the structural plasticity of neurons by quantifying dendritic spine density and morphology in the mPFC using diolistic labeling procedures. It is predicted that administration of CDPPB will increase dendritic spine density, length, head diameter, and overall volume in the mPFC.

mGluR5 PAMs may be viable novel treatments for the cognitive impairments associated with schizophrenia. While mGluR5 PAMs have been tested in several studies assessing reversal of MK-801 induced deficits in cognitive function using operant paradigms (Darrah et al., 2008; Gastambide et al., 2013), no studies to date have examined the importance of pre-treatment and timing between mGluR5 PAM and MK-

801 administration in these paradigms (Experiment 1). In addition, no studies to date have examined the effects of mGluR5 PAMs on extracellular glutamate levels (Experiment 2) or on dendritic spine morphology in the mPFC (Experiment 3), a region critically involved in various aspects of cognition. It is our hope that the culmination of these studies lends further support to the notion that potentiation of mGluR5 function may be a novel therapeutic approach to improving cognitive function in neuropsychiatric diseases such as schizophrenia.

CHAPTER 2

MGLUR5 POSITIVE ALLOSTERIC MODULATION AND ITS EFFECTS ON MK-801 INDUCED SET-SHIFTING IMPAIRMENTS IN A RAT OPERANT DELAYED MATCHING/NON-MATCHING-TO-SAMPLE TASK

Abstract

Rationale Positive allosteric modulators (PAMs) of type 5 metabotropic glutamate receptors (mGluR5) exert pro-cognitive effects in animal models of various neuropsychiatric diseases. However, few studies to date have examined ability of mGluR5 PAMs to reverse cognitive deficits in operant delayed matching/non-matching-to-sample (DMS/DNMS) tasks.

Objectives To determine the ability of the mGluR5 PAM 3-cyano-N-1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB) to reverse set-shifting deficits induced by the NMDA receptor antagonist MK-801.

Methods Male Sprague-Dawley rats were initially trained to lever press for sucrose reinforcement under either DMS or DNMS conditions. Following successful acquisition of the task, reinforcement conditions were reversed (DNMS→DMS or DMS→DNMS). In Experiment 1, rats were treated daily prior to each session with either vehicle/vehicle, vehicle/MK-801 (0.06 mg/kg) simultaneously, CDPPB (20 mg/kg)/MK-801 simultaneously, or CDPPB 30 min prior to MK-801. In Experiment 2, rats were treated with vehicle/vehicle, vehicle/MK-801, or CDPPB 30 min prior to MK-801 only prior to sessions that followed task reversal.

Results In Experiment 1, no group differences in initial task acquisition were observed. Rats treated with vehicle/MK-801 showed significant set-shifting impairments following

task reversal, which were partially attenuated by simultaneous administration of CDPPB/MK-801, and completely precluded by administration of CDPPB 30 min prior to MK-801. In Experiment 2, MK-801 did not impair reversal learning and no other group differences were observed.

Conclusions MK-801 induced deficits in operant set-shifting ability were prevented by pretreatment with CDPPB. MK-801 did not produce deficits in initial task learning or when treatment was initiated following task reversal.

Introduction

Cognitive impairment is a core feature of many chronic illnesses such as schizophrenia, Huntington's disease, Alzheimer's disease, and drug addiction. Cognitive impairments can affect working memory, reference memory, attention, cognitive flexibility, and various other aspects of executive functioning (Green et al. 2004; Silver et al., 2003). Secondary effects of cognitive impairments can affect a patient's ability to maintain relationships, employment, or appropriate daily hygiene practices, and often result in cognitive and behavioral perseveration. Although such impairments and perseveration occur across a range of neuropsychiatric diseases, the focus of the present study was to examine this phenomenon in the context of schizophrenia.

Schizophrenia affects approximately 1% of the population and is typically diagnosed in males during their early 20s and in females during their later 20s. As a result, adequate treatment is necessary for the remainder of the affected individual's life, making schizophrenia one of the most burdensome and costly neuropsychiatric diseases (Rowley et al., 2001). There are several hypotheses regarding the underlying

pathophysiology of schizophrenia, one of which is hypofunctioning of the N-methyl-D-aspartate (NMDA) glutamate receptor subtype (Lin et al., 2012; Olney et al., 1999; Snyder & Gao, 2013). As a result, glutamate-based therapies are currently being explored for the treatment of schizophrenia (Snyder & Gao, 2013; Krystal et al., 2010; Nisewender & Conn, 2010; Menniti et al. 2013). Direct pharmacological targeting of the NMDA receptor via the orthosteric glutamate binding site is generally regarded as a nonviable approach since NMDA receptor agonists produce CNS hyperactivity, seizures, and excitotoxicity. An alternative to directly targeting the orthosteric glutamate binding site is to directly or indirectly target binding sites for obligatory endogenous co-agonists such as glycine and D-serine. For example, inhibition of glycine transporters such as GlyT1 increases extracellular levels of glycine and thereby facilitates NMDA receptor activity (Vandenberg & Aubrey, 2001). Furthermore, various GlyT1 inhibitors have shown efficacy in improving cognitive impairments in schizophrenia (Javitt, 2012).

A third possible approach to increase NMDA receptor function is via activation of type 5 metabotropic glutamate receptors (mGluR5). Facilitation of mGluR5 activity indirectly increases NMDA receptor function through structural and biochemical coupling of these two receptor subtypes. mGluR5 and NMDA receptors are structurally linked via proteins such as postsynaptic density 95 (PSD-95), Shank, and the Homer family of proteins. Localization of these two receptors in close proximity facilitates their bidirectional coupling via signaling intermediates such as phospholipase C and intracellular calcium (Gerber et al., 2007; Hermans & Challiss, 2001; Piers et al., 2012), which increases NMDA receptor activity including increased probability of receptor channel opening (Menniti et al., 2013; Niswender & Conn, 2010). Currently, the most

advantageous approach to increasing mGluR5 receptor function is via positive allosteric modulators (PAMs), which increase mGluR5 signaling only in the presence of endogenous glutamate. The use of orthosteric mGluR5 agonists has proved to be a less viable approach, since these ligands exhibit limited brain penetrance following systemic administration, poor selectivity for specific mGluR subtypes, and induce rapid desensitization of the receptor (Chen & Conn, 2008; Krystal et al., 2010; Menniti et al., 2013; Niswender & Conn, 2010).

Results from several studies indicate that the mGluR5 PAM 3-cyano-N-1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB) reverses experimentally induced cognitive impairments in animal models of schizophrenia. Stefani and Moghaddam (2010) found that CDPPB at doses of 10 and 30 mg/kg attenuated dizocilpine (MK-801) induced cognitive deficits in a T-maze based set-shifting task. Clifton and colleagues (2013) found that acute administration of CDPPB and the mGluR5 PAM ADX47273 reversed phencyclidine (PCP) and MK-801 induced impairment in social novelty discrimination in adult rats, and when administered during adolescence reversed early postnatal MK-801 and PCP-induced impairments. Alternatively, Horio and colleagues (2013) showed that when PCP was administered chronically, acute administration of CDPPB was unable to attenuate deficits in novel object recognition in mice; however, subchronic (14 days) of administration of CDPPB was effective in rescuing such impairments. Uslaner and colleagues (2010) also showed that CDPPB was able to ameliorate MK-801 induced cognitive impairments in novel object recognition, and Vales and colleagues (2010) found that CDPPB was effective in attenuating MK-801 induced impairments in active allothetic place avoidance.

To our knowledge, very few previous studies have examined the ability of mGluR5 PAMs to reverse pharmacologically induced cognitive deficits in an operant set-shifting paradigm (Darrah et al., 2008; Gastembide et al., 2012; Gastembide et al., 2013; Gilmour et al., 2013), and only one utilized CDPPB (Darrah et al., 2008). This distinction is important as Gastembide and colleagues (2012, 2013) found that mGluR5 PAM LSN2463359 was unable to reverse PCP-induced impairments on cognitive flexibility, utilizing a delay component. Operant-based paradigms offer the advantage of modeling tasks used to assess cognitive flexibility and perseveration in humans, such as the Wisconsin Card Sorting Task (Franke et al., 1992; Goldberg et al., 1987; Joel et al., 1997; Silver et al., 2003). In a study by Darrah and colleagues (2008), rats were trained to respond for food reinforcement based on discrimination between two perceptual stimuli dimensions (spatial location of the nosepoke aperture and illumination of a stimulus light), and effects of CDPPB on MK-801 induced deficits in this task were examined. However, this study did not employ a delayed matching/non-matching-to-sample component, which increases working memory load and thus increases the difficulty of the task (Dudchenko, 2004, Dudchenko et al., 2013). In a study by Gilmour and colleagues (2013), rats were trained to respond for food in a delayed non-matching-to-position nose-poke paradigm, and the efficacy of the novel mGluR5 PAMs LSN2463359 and LSN2814617 on reversal of set-shifting performance deficits induced by the competitive (closed channel) NMDA receptor antagonist SDZ 220,581 were assessed. However, in this latter study, the efficacy of these mGluR5 PAMs against the effects of more widely used non-competitive (open channel) NMDA antagonists such as MK-801 were not assessed. In addition, both of these studies utilized acute dosing procedures which can

lead to difficulty in interpretation of the potential antipsychotic and pro-cognitive effects of these ligands when administered daily in a clinical setting (Hagan & Jones, 2005; Varvel et al., 2002).

Therefore, the purpose of the present study was to assess the ability of CDPPB to reverse MK-801 induced cognitive deficits in an operant set-shifting task incorporating delayed matching/non-matching-to-sample procedures (Experiment 1). Chronic drug administration was utilized in order to more closely resemble daily intake patterns that are generally required for therapeutic purposes in humans. However, to control for the possibility that daily administration of CDPPB and/or MK-801 during the first phase of testing might have carryover effects on performance following task reversal, separate groups of animals received drug administration only during the post-reversal phase of the procedures (Experiment 2). Finally, many of the aforementioned studies have assessed the ability of CDPPB to reverse MK-801 induced cognitive deficits utilizing vastly different drug administration paradigms, with some studies administering CDPPB prior to MK-801, some administering the two drugs simultaneously, and others administering MK-801 prior to CDPPB. Therefore, a tertiary purpose of the present study was to establish whether pretreatment with CDPPB prior to MK-801 would produce more robust effects on set-shifting ability deficits as compared to simultaneous administration.

Methods and Materials

Subjects

Subjects were male Sprague-Dawley rats (Harlan Laboratories, Livermore, CA) weighing 250-300 g upon arrival. Rats were pair-housed throughout the duration of the study. The vivarium was held at a temperature of 22±1°C, and a 12 hour reversed light/dark cycle (lights off 7 am) was used. Animals had free access to water throughout the experiment except during behavioral testing. Rats were food restricted to 80% of their body weight by allowing them to free-feed for one hour after each daily session. Food restriction was necessary since it has previously been demonstrated that MK-801 decreases the reinforcing properties of sucrose in free feeding animals (Vardigan et al., 2010), which would adversely affect task performance in the present study. In addition, food reinforcement was not used since it has previously been demonstrated that MK-801 can actually increase food intake and meal size under certain conditions (Burns & Ritter, 1997; Gillespie et al., 2005; Treece et al., 2000), thus confounding the use of food as a reinforcer in during behavioral testing. Experimental group sample sizes were as follows: vehicle/vehicle (n=8), vehicle/MK-801 (n=6), CDPPB/MK-801 simultaneously (n=7), and CDPPB 30 min prior to MK-801 (n=9). Animals were maintained in accordance with the guidelines described in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and all procedures and facilities were approved by the Institutional Animal Care and Use Committee at Arizona State University.

Drug Treatment

3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB) was synthesized by Chemir Analytical Services (Maryland Heights, MO) according to published methods (Kinney et al., 2005) and suspended in a vehicle consisting of 10% v/v Tween 80 (Sigma-Aldrich, St. Louis, MO). MK-801 was purchased from Sigma-Aldrich and dissolved in sterile saline (0.9% w/v NaCl). Doses for CDPPB (20 mg/kg) and MK-801 (0.06 mg/kg) were based on previous studies (Ayala et al., 2009; Horio et al., 2013; Stefani & Moghaddam, 2010; Uslaner et al., 2009) and pilot work performed in our laboratory. All drugs were administered via the subcutaneous (s.c.) route in a volume of 0.5 ml.

Delayed Matching/Non-Matching-to-Sample Paradigm

Delayed match-to-sample (DMS) or non-match-to-sample (DNMS) tasks were based on previously published procedures (Joel et al., 1997) and were conducted in daily sessions (approx. 45 min in length) consisting of 30 trials per session. Each trial was comprised of three components: a sample component, choice component and an inter-trial interval (ITI). At the beginning of each daily session, animals were placed into standard operant conditioning chambers (model ENV-007, Med Associates, St. Albans, VT) containing a house light, food receptacle and two retractable levers. Each chamber was interfaced to a PC computer, and MED-PC IV software (Med Associates) was used to control experimental parameters and record responses. The house light was illuminated to signal the beginning of the first trial. During the sample component, either the left or right lever, designated the sample lever, was randomly inserted into the chamber. Pressing the sample lever resulted in delivery of a sucrose pellet reinforcer (45 mg, Test

Diet, St. Louis, MO) and a 30 sec delay. Following the delay, the choice component of the task commenced wherein both levers were inserted into the chamber. During the choice component, if the animal was assigned to the DMS condition as the initial task and pressed the same lever that was presented during the sample component, a sucrose pellet was delivered followed by a 30 sec inter-trial interval (ITI). An incorrect lever press resulted in the house-light turning off and initiation of a 60 sec time-out period. Alternatively, if the animal was assigned to the DNMS condition for the initial task and pressed the lever that was not presented during the sample component, a sucrose pellet was delivered followed by a 30 sec ITI. To control for task-specific learning, half of the subjects in each group were assigned to the DMS condition as the initial task and the other half the DNMS. Animals were considered to have met acquisition criteria when they exhibited at least 80% correct responses for 4 consecutive days, and were allowed a maximum of 21 days of testing to meet this criterion. In the event animals reached this criterion prior to 21 days of testing, they continued in the same testing conditions through the 21st day of testing. Animals that did not reach the 80% correct acquisition criteria by the 21st day of testing were removed from the study. On Day 22 of testing, animals were switched from their previously assigned task to the opposite task (i.e., DNMS→DMS, or DMS→DNMS) and subsequently tested for 21 additional days. Animals were then euthanized by anesthesia with isoflurane followed by decapitation and brain removal.

Testing Procedures

In Experiment 1, animals were randomly assigned to one of the following 4 treatment groups and were treated 20 min prior to each daily session with the following

drug combinations: vehicle/vehicle, vehicle/MK-801 (0.06 mg/kg) simultaneously, CDPPB (20 mg/kg)/MK-801 simultaneously, or CDPPB 30 min prior to MK-801. In order to control for the possibility that daily administration of CDPPB and/or MK-801 during the first phase of testing (prior to task reversal) might have carryover effects on performance following task reversal, Experiment 2 was conducted, in which separate groups of animals underwent similar initial testing, but received either vehicle/vehicle, vehicle/MK-801, or CDPPB 30 min prior to MK-801 only during the post-reversal phase of the procedures (days 22-42).

Data Analyses

For each daily session, the proportion of correct responses (calculated as the total number of correct responses divided by the overall total number of responses) were analyzed using a mixed two-way ANOVA with Treatment as the between-subjects factor and Day (session) as the within-subjects factor. Since initial analyses revealed no group differences during the acquisition of the initial task prior to task reversal for both Experiments 1 and 2, only data from sessions following task reversal were included in subsequent analyses. Multivariate ANOVAs were used to determine differences between specific treatment groups on each day of testing following task reversal. Statistical analyses were performed using Prism (version 5.00, GraphPad Software, La Jolla, CA) and SPSS (version 21.0, IBM, Armonk, NY). P-values less than 0.05 were considered statistically significant for all tests.

Results

Each experimental group had an initial sample size of n=10 animals per group, half of which were initially assigned to the DMS condition and the other half to the DNMS condition. However, in Experiment 1, the following numbers of animals failed to meet acquisition criteria by the 21st day of the study, and were therefore removed from the experiment: vehicle/vehicle (n=2), vehicle/MK-801 (n=4), CDPPB/MK-801 simultaneously (n=3), and CDPPB 30 min prior to MK-801 (n=1). Therefore, the final group sample sizes were as follows: vehicle/vehicle (n=8), vehicle/MK-801 (n=6), CDPPB/MK-801 simultaneously (n=7), and CDPPB 30 min prior to MK-801 (n=9). There were no apparent group biases in failure to acquire the task, such that roughly equal numbers of animals in each group acquired the initial DMS or DNMS task. In Experiment 2, all 10 animals per group reached acquisition criteria during the initial phase of the experiment.

Data from all 4 treatment groups in Experiment 1 are shown in Fig. 1A. For ease of visualization of inter-group comparisons, data from various treatment groups in Fig. 1A are re-plotted in Figs. 1B, 1C and 1D. No group differences were observed during the initial task acquisition (days 1-21) (all p-values >0.05). Following task reversal (days 22-42), a mixed two-way ANOVA revealed significant effects of Treatment ($F_{3,26}$=5.21, $p<0.01$), Day ($F_{20,520}$=170.95, $p<0.001$) and a Treatment x Day interaction ($F_{60,520}$=2.04, $p<0.001$). Post hoc analysis revealed that animals in the vehicle/MK-801 group exhibited a significant decrease in proportion of correct responding as compared with the vehicle/vehicle group on days 29-35, 37, and 39-41 (all p's<0.05), indicating that MK-801 induced a significant impairment in set-shifting ability (Fig. 1B). Animals pre-treated

with CDPPB 30 min prior to MK-801 (CDPPB 30 min/MK-801, Fig. 1C) showed a significantly increased proportion of correct responding as compared with animals treated with vehicle/MK-801 group on days 29-35, 37, and 39-41 (all p's<0.05), indicating a reversal of MK-801 induced deficits. Furthermore, pre-treatment with CDPPB prior to MK-801 resulted in performance equivalent to vehicle/vehicle treated rats (all p's >0.05, see Fig. 1D). However, simultaneous treatment with CDPPB and MK-801 produced only partial attenuation of MK-801 induced deficits in set-shifting ability, as compared to the vehicle/MK-801 group (Fig. 1E), as evidenced by a significantly increased proportion of correct responding only on day 33 (p<0.05), and a trend towards significant differences on days 31, 34, and 41 (p's=0.08, 0.07, and 0.06, respectively).

In Experiment 2, no group differences were observed in acquisition of the initial task (all p's>0.05). Following task reversal (Fig. 1F), a mixed two-way ANOVA revealed a significant effect of Day ($F_{20,567}$=70.87, p<0.001) but no significant effects of treatment (p>0.05) or a Treatment x Day interaction (p>0.05). These results suggest that MK-801 does not induce impairments in set-shifting ability when treatment is initiated following task reversal, and that are no significant effects of CDPPB on set-shifting ability.

Discussion

In this study, we demonstrated that pharmacological blockade of NMDA receptors with MK-801 produced deficits in a delayed matching/non-matching-to-sample operant set-shifting task, and these effects were reversed when the mGluR5 PAM CDPPB was administered 30 minutes prior to, but not simultaneously with, MK-801. While other studies have reported similar positive effects of mGluR5 PAMs on the

reversal of deficits in operant set-shifting paradigms induced by NMDA receptor antagonists (Darrah et al., 2008; Gilmour et al., 2013), it should be emphasized that the current study, as well as that conducted by Gilmour and colleagues (2013), employed a delayed matching/non-matching-to-sample component, which increases working memory load and can be considered to be of increased translational value in the assessment of the efficacy of novel antipsychotic medications (Dudchenko, 2004; Dudchenko et al., 2013). In addition, most other studies (summarized below) examining mGluR5 PAM-induced reversal of the cognitive impairing effects of MK-801 have used either acute or subchronic dosing regimens, whereas in the present study animals received pharmacological treatments either throughout the course of the study (Experiment 1) or only during the post-reversal phase (Experiment 2). As suggested by others (Hagan & Jones, 2005; Varvel et al., 2002), we assert that chronic dosing regimens such as those used in the present study have a higher translational value for assessing the efficacy of novel cognition-enhancing or antipsychotic medications, which would most likely require daily dosing in humans.

The ability of CDPPB to reverse MK-801 induced deficits in set-shifting ability is consistent with other findings indicating reversal of pharmacologically induced cognitive effects of mGluR5 PAMs using non-delay based operant tasks (Darrah et al., 2008); spatial alternation tasks (Fowler et al., 2013; Stefani & Moghaddam, 2010); social novelty discrimination (Clifton et al., 2013); novel object recognition (Horio et al., 2013; Reichel et al, 2011; Uslaner et al., 2009); and active allothetic place avoidance (Vales et al., 2009). mGluR5 PAMs have also been shown to reverse MK-801 induced impairments in sucrose preference (Vardigan et al., 2010), taste aversion and inhibitory

avoidance tasks (Fowler et al., 2011), and alterations in prefrontal cortex neuronal firing patterns (Lecourtier et al., 2007; Homayoun et al., 2008; Homayoun & Moghaddam, 2010). In unimpaired animals, mGluR5 PAMs have been shown to improve spatial and recognition memory abilities (Ayala et al., 2009; Balschun et al., 2006; Uslaner et al., 2009). Thus, mGluR5 PAMs appear to be a promising class of cognition-enhancing agents, although recent reports of neurotoxicity induced by high dose exposure to these compounds requires further investigation (Parmentier-Batteur et al., 2013).

The more robust efficacy of CDPPB pretreatment 30 min prior to MK-801 as compared with simultaneous administration of these two ligands is likely attributable to one or more factors. One possibility is that CDPPB may be slower to enter the brain than MK-801. In support of this, Kinney and colleagues (2005) demonstrated that acute CDPPB administration produces a brain-to-plasma area under the curve (AUC) ratio of 1.7, while Vezzani and colleagues (1989) showed that MK-801 produces a brain-to-plasma AUC of 12.5, suggesting significantly improved brain penetration of MK-801 vs. CDPPB. Another possibility is that CDPPB is less able to reverse the cognitive effects of MK-801 when this latter ligand is already bound to the NMDA receptor. This latter possibility may seem unlikely in light of findings by Stefani and Moghaddam (2010), which showed that treatment of rodents with similar doses of MK-801 *prior* to CDPPB prevented MK-801 induced deficits on cognitive set-shifting ability in a spatial plus maze task. However, in this study, both drugs were administered acutely rather than chronically as in the present study, and thus the order of ligand administration may become more important when these ligands are given repeatedly. Another possible explanation for the

improved efficacy of CDPPB, when administered 30 min prior to MK-801, as opposed to simultaneously may lie within the mechanism of action of MK-801.

Worthy of discussion is the fact that recent findings suggest that there are different functional classes of mGluR5 PAMs that can exert differential effects on mGluR5 receptor function and the ability to reverse cognitive or behavioral deficits induced by NMDA receptor antagonists. For example, it has been reported that newer mGluR5 PAMs such as LSN2463359 and LSN2814617 are able to reverse decrements in instrumental responding for food as well as reversal learning in a digging-based and delayed match-to-position food seeking tasks induced by the competitive (closed channel) NMDA receptor antagonist SDZ 220,581 (Gilmour et al., 2013). Surprisingly, LSN2463359 failed to reverse performance decrements in these tasks induced by the non-competitive (open channel) NMDA receptor antagonists MK-801 and PCP (Gastambide et al., 2013). However, it should be noted that these studies only evaluated the acute effects of these mGluR5 PAMs. Ligand binding and pharmacokinetic experiments in these studies revealed very different profiles of these newer mGluR5 PAMs as compared to CDPPB, such that increased brain penetrance, receptor affinity, and binding to an allosteric site on the mGluR5 receptor are different from that of CDPPB. Importantly, it has been suggested that mGluR5 PAMs acting on separate allosteric binding sites on the receptor recruit different signal transduction mechanisms, with some allosteric sites inducing increased intracellular calcium mobilization as compared to activation of extracellular signal-related kinase 1/2 (ERK1/2), and vice versa (Zhang et al., 2005). These different binding profiles and subsequent engagement of different cellular signaling mechanisms may ultimately influence their ability to indirectly potentiate

NMDA receptor function when the receptor is in an open or closed state. Thus, the ability of mGluR5 PAMs to attenuate or reverse cognitive and behavioral impairments that are induced by NMDA receptor blockade may be highly dependent on the molecular profile of each ligand used, as well as the dosing regimen and behavioral paradigm employed. Future studies are necessary to determine the precise cellular signaling mechanisms underlying the effects observed in the present study.

Finally, another finding from the present study is that MK-801 does not induce impairments in the acquisition of set-shifting ability when MK-801 treatment is initiated following task reversal. These observations are consistent with various bodies of literature suggesting that impaired NMDA receptor function at low to moderate doses does not lead to deficits in initial task learning (Chadman et al., 2006; Harder et al., 1998; Murray et al., 1995; Palencia & Ragozzino, 2004; van der Meulen et al., 2003; van der Staay et al., 2011; Wozniak et al., 1990), but appears to have more deleterious effects on set-shifting that result in behavioral and cognitive perseveration (Braff et al., 1991; Egerton et al., 2005; Franke et al., 1992; Goldberg et al., 1987; Green et al., 2004; Silver et al., 2003). However, it could be argued that in Experiment 1 of the present study, the behavioral effects of CDPPB and/or MK-801 following task reversal could have been a result of accumulation of either drug during chronic drug administration prior to task reversal. The plasma elimination half-lives of CDPPB in rats are approximately 4 and 2 hrs, respectively (Kinney et al., 2005; Vezzani et al., 1989), and with chronic administration such an accumulation could indeed occur. However, several observations in the present study argue against this possibility. First, should any behavioral effects of CDPPB be carried over from the pre-reversal to the post-reversal phase, these effects

would likely have been observed in both the CDPPB/MK-801 and CDPPB 30 min/MK-801. However, this is doubtful since Experiment 1 demonstrated a reversal of the effects of MK-801 only in animals receiving CDPPB 30 min prior to MK-801 (Fig. 1A and 1C). Also, initiation of all drug treatments following task reversal (Fig. 1F) did not produce any deficits in acquiring the new task, suggesting a lack of carryover effects of MK-801 from the first phase of the experiment to the second phase. Finally, it has previously been demonstrated that long-term cognitive impairing effects by chronically administered MK-801 are predominantly observed at high doses (i.e., 0.2 mg/kg; Li et al., 2011) and not at the dose used in the present study (0.06 mg/kg). Taken together, these observations suggest that the ability of MK-801 to induce deficits in set-shifting ability, and its reversal by pretreatment with CDPPB, are mediated by continued drug administration following task reversal.

In summary, the present study lends further support to the notion that potentiation of mGluR5 function may be a novel therapeutic approach to improving cognitive deficits involving NMDA hypofunction, especially those involving higher working memory loads (Krystal et al., 2010; Niswender & Conn 2010; Menniti et al., 2013; Snyder & Gao, 2013). Also, our findings support previous research showing that low to moderate doses of MK-801 selectively impair reversal learning and behavioral perseveration, but do not impair initial task learning. This is consistent with the clinical literature where studies have shown that patients with schizophrenia or otherwise impaired prefrontal function are able to learn an initial cognitive task, but set-shifting ability and perseveration following a change in response contingency requirements is dramatically impaired (Braff et al., 1991; Egerton et al., 2005; Franke et al., 1992; Goldberg et al., 1987; Green et al., 2004;

Silver et al., 2003). The present study also identifies the necessity of pretreatment with an mGluR5 PAM prior to administration of a non-competitive NMDA antagonist under chronic dosing conditions. Future studies examining the precise cellular signaling mechanisms downstream of mGluR5 receptor potentiation in producing the pro-cognitive effects are needed. Additional studies are also needed to explore the ability of novel mGluR5 PAMs with improved brain penetrance and receptor affinity to reverse cognitive deficits induced by closed channel NMDA antagonists, as well as in other genetic or neurodevelopmental models of cognitive dysfunction. In addition, future studies should also employ additional behavioral paradigms that assess other cognitive domains such as spatial memory, attention, and sensorimotor gating.

CHAPTER 3

MGLUR5 POSITIVE ALLOSTERIC MODULATION REVERSES MK-801 INDUCED INCREASES IN EXTRACELLULAR GLUTAMATE LEVELS IN THE RAT MEDIAL PREFRONTAL CORTEX

Abstract

Rationale Positive allosteric modulators (PAMs) of type 5 metabotropic glutamate receptors (mGluR5) exert antipsychotic-like and pro-cognitive effects in animal models of schizophrenia and drug addiction, and can reverse cognitive deficits induced by NMDA receptor antagonists. However, it is currently unknown if mGluR5 PAMs can modulate NMDA receptor antagonist-induced alterations in extracellular glutamate levels in the medial prefrontal cortex (mPFC).

Objectives To determine the ability of the mGluR5 PAM 3-cyano-N-1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB) to reduce elevated extracellular glutamate levels induced by the NMDA receptor antagonist dizocilpine (MK-801) in the mPFC.

Methods Male Sprague-Dawley rats were treated for ten consecutive days with either CDPPB (20 mg/kg s.c.) alone or in combination with MK-801 (0.06 mg/kg s.c.), or their corresponding vehicles. On the final day of treatment, *in vivo* microdialysis was performed in the mPFC and samples were collected every 30 min for monitoring of extracellular glutamate levels.

Results Compared to animals receiving only vehicle, administration of MK-801 significantly increased extracellular levels of glutamate in the mPFC. This effect was not observed in animals that were co-administered with CDPPB. Administration of CDPPB

alone had no evidence of an effect on extracellular glutamate levels in the mPFC as compared to animals treated only with vehicle.

Conclusions The mGluR5 PAM CDPPB reverses increases in extracellular glutamate in the mPFC induced by MK-801. These findings provide neurochemical support for the ability of mGluR5 PAMs to reverse NMDA antagonist-induced cognitive deficits, which may underlie the mechanisms of these ligands to produce pro-cognitive effects in disorders such as schizophrenia and drug addiction.

Introduction

Cognitive impairments are common in disorders such as schizophrenia and drug addiction, and can affect working memory, reference memory, attention, and executive functioning (Green et al., 2004; Kalivas & Volkow, 2005; Panenka et al., 2013; Silver et al., 2003; Stavro et al., 2013). Phencyclidine (PCP) and drugs such as MK-801 noncompetitively block N-methyl-D-aspartate (NMDA) type glutamate receptors and induce cognitive impairments closely resembling those observed in schizophrenia (Allen & Young, 1978; Javitt & Zukin, 1991; Krystal et al., 2003). Consequently, it is suggested that dysregulation of NMDA receptors (NMDARs) may be involved in the pathophysiology of schizophrenia (Coyle et al., 2012; Gilmour et al., 2012; Zhou & Sheng, 2013). Of the many glutamatergic theories of schizophrenia, the NMDAR hypofunction theory suggests that dysregulation of glutamate neurotransmission is caused by hypofunctional NMDAR on subcortical GABAergic interneurons which disinhibit cortical glutamatergic efferent pathways, resulting in a hyperglutamatergic state (Marek et al., 2010). In addition, NMDA antagonism is a mechanism of action of various drugs

of abuse including alcohol and dissociative hallucinogens (Lovinger et al., 1989; Vollenweider, 2001), and chronic intake of various drugs of abuse alters synaptic glutamate homeostasis, the expression and function of various glutamate receptors, and extracellular levels of glutamate in various brain regions (Gass & Olive, 2008; Kalivas, 2009).

Glutamate is the most prevalent excitatory neurotransmitter in the central nervous system, mediating as much as 70% of both fast and slow excitatory neurotransmission (Gass & Olive, 2008). Glutamate receptors are either ligand-gated ion channels (i.e., ionotropic glutamate receptors, or iGluRs) or G-protein coupled receptors (i.e., metabotropic glutamate receptors, or mGluRs). iGluRs are ion channels that are permeable to cations, and function by allowing Na^+ and Ca^{2+} to enter the cell initiating action potentials and activating various intracellular signaling pathways. iGluRs are divided into three different subtypes, NMDA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), and kainic acid (KA) (Gass & Olive, 2008). mGluRs mediate slower, modulatory neurotransmission, and based on their intracellular signaling and pharmacological properties, are categorized into three families. Group I mGluRs consist of mGluR1 and mGluR5 receptors, Group II mGluRs consist of mGluR2 and mGluR3 receptors, and Group III consist of mGluR4, mGluR6, mGluR7 and mGluR8 receptors (Gass & Olive, 2008; Olive, 2009). For the purposes of the present study, we will focus our discussions on Group I mGluRs.

Group I mGluRs primarily utilize $G_{q/11}$ signaling mechanisms which activate phospholipase C to form diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG activates protein kinase C (PKC), while IP_3 exerts further downstream effects by binding

to IP_3 receptors on the endoplasmic reticulum, releasing intracellular Ca^{2+} which in turn activates other intracellular messengers such as calcium/calmodulin-dependent kinase II (CaMKII). PKC exerts further downstream effects via phosphorylation of cAMP response element-binding protein (CREB) and other signaling molecules which can eventually lead to altered gene transcription (Hermans & Challiss, 2001). Group I mGluRs, particularly mGluR5, are physically linked to NMDARs by various scaffolding proteins (such as Homer, Shank and GKAP), and positively modulate NMDAR function. Through its activation of PKC, mGluR5 activation indirectly increases the phosphorylation of NMDAR subunits, thereby indirectly increasing the probability of NMDAR channel opening (Niswender & Conn, 2010). Due to the postsynaptic localization of Group I mGluRs, these receptors can initiate and regulate long-term potentiation (LTP) and long-term depression (LTD) by postsynaptic mechanisms such as potentiation of NMDAR function (Anwyl, 1999; Bellone et al., 2008; Gladding et al., 2009). These structural and biochemical interactions allow for the indirect activation of NMDARs while reducing the incidence of excitotoxicity (Albensi, 2007; Hardingham & Bading, 2003).

Positive allosteric modulators (PAMs) of glutamate receptors are ligands that facilitate receptor functioning in the presence of endogenous glutamate binding to the orthosteric binding site. Various studies have demonstrated that mGluR5 PAMs such as 3-cyano-N-1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB) reverse cognitive impairments induced by NMDA antagonists. For example, Stefani and Moghaddam (2010) found that CDPPB at doses of 10 and 30 mg/kg attenuated MK-801 induced cognitive deficits in a T-maze based set-shifting task. Alternatively, Horio and colleagues

(2013) showed that when PCP was administered chronically, acute administration of CDPPB was unable to attenuate deficits in novel object recognition in mice; however, subchronic (14 days) of administration of CDPPB was effective in rescuing such impairments. Uslaner and colleagues (2009) also showed that CDPPB ameliorated MK-801 induced impairments in a novel object recognition task. Other studies have demonstrated the ability of mGluR5 PAMs to reverse pharmacologically induced cognitive deficits in an operant set-shifting paradigm (Darrah et al., 2008; Gilmour et al., 2013; LaCrosse et al., 2014). Finally, our laboratory has shown that mGluR5 PAMs have pro-cognitive effects of in animal models of addiction, such as facilitation of extinction learning (Cleva et al., 2011; Kufahl et al., 2013; Gass et al., 2014), which are mediated at least in part by sub-cortical regions of the mPFC (Gass et al., 2014), and reversal of methamphetamine-induced deficits in object recognition (Reichel et al., 2011).

Various *in vivo* microdialysis studies have shown that administration of MK-801 or other NMDA antagonists increases extracellular levels of glutamate in the mPFC, and these effects can be reversed by traditional antipsychotics (Adams & Moghaddam, 2001; Lena et al., 2006; Lopez-Gil et al., 2007, 2009; Lorrain et al., 2003; Moghaddam et al., 1997; Pietraszek et al., 2009; Roenker et al., 2011; Roenker et al., 2012; Zuo et al., 2006). However, studies examining the effects of mGluR5 PAMs on extracellular neurotransmitter levels are very sparse, and of the few that have been published, they have generally focused on changes in extracellular levels of dopamine (Liu et al., 2008; Lecourtier et al., 2007). Given that mGluR5 PAM reverse MK-801 and other pharmacologically induced cognitive deficits, and the effects of mGluR5 PAMs on extracellular glutamate levels are largely unknown, the purpose of this study was to

examine the effects of the mGluR5 PAM CDPPB on extracellular glutamate levels in the mPFC in naive and MK-801 treated rodents.

Methods and Materials

Subjects

Subjects were male Sprague-Dawley rats (Harlan Laboratories, Livermore, CA) weighing 250-300 g upon arrival. Rats were pair-housed until surgical procedures, after which they were single housed for the duration of the study. The colony room was held at a temperature of 22±1°C, and a 12 hour reversed light/dark cycle (lights off 7 am) was used. Food and water were available to the animals *ad libitum* throughout the experiment. Animals were maintained in accordance with the guidelines described in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and all procedures and facilities were approved by the Institutional Animal Care and Use Committee at Arizona State University.

Surgery

For stereotaxic implantation of guide cannula, rats were anesthetized with isoflurane (2%) at a flow rate of 2 L/min. After anesthesia was obtained, rats were mounted in a stereotaxic frame (Stoelting, Wood Dale, IL), and the incisor bar was set at 3.0 mm. A stainless guide cannula (21-gauge, Synaptech, Marquette, MI) equipped with dummy probe was unilaterally aimed at the medial PFC (anterior +3.2, lateral +0.6, and vertical – 2.2 mm relative to bregma and skull surface; Paxinos & Watson, 2007).

Cannulae were secured to the skull with stainless steel skull screws and cranioplastic cement, and animals were allowed to recover for at least 2 days prior to commencement of drug treatment.

Drug Treatments

The mGluR5 positive allosteric modulator 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB) was synthesized by Chemir Analytical Services (Maryland Heights, MO). CDPPB was dissolved in 10% v/v Tween 80 (Sigma-Aldrich, St. Louis, MO) and administered at a dose of 20 mg/kg, and MK-801 (Sigma-Aldrich) was dissolved in saline and administered at a dose of 0.06 mg/kg. All rats received daily injections of CDPPB and/or MK-801 or their corresponding vehicles via the subcutaneous (s.c.) route in a volume of 0.5 mL for ten consecutive days. Animals were divided into 4 treatment groups and were treated for 10 days with the following drug combinations: vehicle/vehicle, vehicle/CDPPB, CDPPB/MK-801, and vehicle/MK-801. The first injection (CDPPB or vehicle) was given 30 min prior to the second injection (MK-801 or saline). Doses of CDPPB and MK-801 were chosen based on previous studies by our laboratory and others (Stefani & Moghaddam, 2010; Kufahl et al., 2013).

Microdialysis Procedures

On the 9th day of drug treatment, animals were lightly anesthetized with isoflurane, dummy probes removed, and microdialysis probes equipped with 2 mm cuprophane membranes (20 kDa cut-off weight, outer diameter 0.36 mm, Synaptech) were implanted into the mPFC. Rats were then housed overnight in cylindrical

microdialysis cages equipped with dual channel liquid swivels mounted onto counterbalanced lever arms (Instech Laboratories, Plymouth Meeting, PA). Animals were provided with food and a water bottle. Probes were perfused overnight with artificial cerebrospinal fluid (aCSF) containing (125 mM NaCl, 2.5 mM KCL, 0.5 mM $NaH_2PO_4H_2O$, 5 mM Na_2HPO_4, 1 mM $MgCl_2 \bullet 6H_2O$, 5 mM D-glucose, 1.2 mM $CaCl_2 \bullet 2H_2O$, pH-7.3-7.5) at a flow rate of 0.05 µl/min. The following morning, the flow rate was increased to 1.5 µl/min and samples were collected every 30 min. After collection of 4 baseline samples, the final pretreatment of CDPPB or vehicle injection was given, followed 30 min later by MK-801 or vehicle. A total of 5 post-injection samples were collected. At the end of sample collection, rats were deeply anesthetized with isoflurane and euthanized by decapitation. Brains were extracted, cut on a cryostat, stained with cresyl violet, and verified for correct microdialysis probe placement.

HPLC Analysis of Glutamate Levels

Glutamate was quantified in dialysate samples using isocratic high-performance liquid chromatography with fluorescence detection. Samples were stored in the cooling tray (8°C) of a Shimadzu SIL-10AF autosampler and derivatized in a 3 min reaction with the addition of 30 µl of a solution containing o-phthaldehyde (10 mg %; dissolved first in 1 ml 100% methanol and added to 29 ml of OPA Diluent (Pickering Laboratories, Mountain View, CA)) and mixed with 100% β-mercaptoethanol in a 1:2000 ratio. Sample separation was accomplished using an ESA HR-80 × 3.2 column with 120 µm pore size (ESA, Chelmsford, MA). The mobile phase was 0.1 M dibasic sodium phosphate (pH 6.75) mixed with 28% methanol and continuously pumped at 0.5 ml/min

using a Shimadzu LC-20AD Prominence pump. A Shimadzu RF-10XL Fluorescence Detector was set with an excitation wavelength of 348 nm and an emission wavelength of 450 nm. Glutamate concentrations were determined against external standards using peak heights determined by LabSolutions Software (Shimadzu, LC solution Version 1.22 SP1). The limit of detection for this assay was ~25 nM and the limit of quantification was ~135 nM.

Data Analyses

For assessment of baseline differences in dialysate glutamate content, the average dialysate glutamate levels in the 4 baseline samples were compared across treatment groups using a one-way ANOVA. For the purposes of visualization of time course of drug effects, all glutamate values were converted to a percent baseline using the calculated raw baseline values for each rat. For assessment of drug effects, raw dialysate glutamate content was converted to area under the curve (AUC) for the 5 post-injection samples (Gardier, 2013; Maskos et al., 2005; Mitsushima et al., 2013). For each animal, post-injection AUC values were calculated via trapezoidal approximation as AUC = $0.5(C_1+C_2)(t_2-t_1) + 0.5(C_2+C_3)(t_3-t_2) + 0.5(C_3+C_4)(t_4-t_3)$…., where C_X is the dialysate glutamate concentration (C, in nM) in post-injection sample X, and t_X is the time (in min) at the beginning of post-injection sample X. Time of injection was considered t=0. A one-way ANOVA was used to determine overall group differences followed by post-hoc Newman-Keuls multiple comparison rests. Statistical analyses were performed using GraphPad Prism (version 5.00, GraphPad Software, La Jolla, CA).

Results

Each experimental group had an initial sample size of n=10 animals per group. However, due to improper probe placement or microdialysis probe patency issues during sample collection, the following numbers of animals were removed from the experiment: vehicle/vehicle (n=3), CDPPB/vehicle (n=2), CDPPB/MK-801 (n=4), and vehicle/MK-801 (n=4). Therefore, the final groups sample sizes were as follows: vehicle/vehicle (n=7), CDPPB/vehicle (n=8), CDPPB/MK-801 (n=6), and vehicle/MK-801 (n=6). The shaded area in Figure 2A indicates the region of the mPFC that was defined for proper placement of microdialysis probes.

Figure 2B shows the time course of drug effects in all four treatment groups, expressed as a percent of baseline levels of glutamate. As shown in Figure 2C, no significant differences were observed in AUC values for baseline (samples 1-4) between treatment groups ($F(3,23)=2.406$, $p>0.05$). Analyses of post-injection AUC values (samples 5-9, Figure 2D) revealed a significant effect of treatment group ($F(3,23)=7.282$, $p<0.005$). Post hoc analyses revealed that post-injection AUC values were significantly elevated in the vehicle/MK-801 group as compared to the vehicle/vehicle group ($p<0.01$), indicating that MK-801 produced an elevation in extracellular levels of glutamate in the mPFC. Post-injection AUC values in the CDPPB/MK-801 group were significantly lower than those in the vehicle/MK-801 ($p<0.05$), indicating a reversal of MK-801 induced increases in extracellular glutamate by pretreatment with CDPPB. These observations were further supported by a lack of significant differences in post-injection AUC values between the vehicle/vehicle and CDPPB/MK801 groups ($p>0.05$). Post-injection AUC values were not significantly different between the vehicle/vehicle and CDPPB/vehicle

groups, suggesting that administration of CDPPB alone does not alter extracellular levels of glutamate in the mPFC.

Discussion

The primary findings of this study were that pretreatment with CDPPB significantly attenuated MK-801 induced increases in extracellular glutamate in the mPFC. However, administration of CDPPB alone showed no evidence of an effect on extracellular glutamate levels. Given that CDPPB and other mGluR5 PAMs have been shown to reverse pharmacologically induced performance deficits in prefrontal-mediated cognitive tasks such as set-shifting (Stefani & Moghaddam, 2010; Gilmour et al., 2013; Horio et al., 2013; Darrah et al., 2008; LaCrosse et al., 2014), our findings may provide a potential neurochemical correlate of the pro-cognitive effects of mGluR5 PAMs. These findings are consistent with those from electrophysiological studies, where it has been demonstrated that CDPPB prevents MK801-induced increases in spontaneous activity of pyramidal neurons in the mPFC, and also normalizes MK-801 induced disruption in burst activity of these neurons (Lecourtier et al., 2007). Thus, positive allosteric modulation of mGluR5 function may be a mechanism by which to restore disrupted excitatory neurotransmission in the mPFC that is observed in neuropsychiatric disorders such as schizophrenia and drug addiction.

In our study, we utilized a chronic (10 day) treatment regimen to more closely model typical daily dosing patterns in humans. We found no significant differences in basal levels of extracellular glutamate levels across all four treatment groups. This was somewhat surprising given the fact that acute treatment with MK-801 or NMDA

antagonists have been shown to increase extracellular glutamate levels in the mPFC (Adams & Moghaddam, 2001; Lena et al., 2006; Lopez-Gil et al., 2007, Lopez-Gil et al., 2009; Lorrain et al., 2003; Moghaddam et al., 1997; Pietraszek et al., 2009; Roenker et al., 2011; Roenker et al., 2012; Zuo et al., 2006), and we expected to observe higher basal levels of extracellular glutamate in the vehicle/MK-801 group as compared to the vehicle/vehicle group. Alternatively, Zuo and colleagues (2006) demonstrated that repeated administration of MK-801 (7 days) actually reduced extracellular levels of glutamate in the mPFC, while acute MK-801 administration increased extracellular glutamate levels. However, it should be noted that this study used a much higher dose of MK-801 (0.6 mg/kg), therefore it is possible that repeated administration of the lower dose used in the present study (0.06 mg/kg) did not lead to the same neuronal adaptations that alter the directionality of MK-801 induced changes in extracellular glutamate levels in the mPFC. In addition, baseline samples were collected in the morning following the 9^{th} injection and prior to the 10^{th} injection, and thus any MK-801 induced increases that may have occurred on day 9 would likely have dissipated by the time baseline samples were collected. Regardless, the observed MK-801 induced increase in extracellular glutamate levels on day 10 are consistent with numerous other reports showing similar increases following acute administration (Adams & Moghaddam, 2001; Lena et al., 2006; Lopez-Gil et al., 2007, 2009; Lorrain et al., 2003; Moghaddam et al., 1997; Pietraszek et al., 2009; Roenker et al., 2011; Roenker et al., 2012; Zuo et al., 2006).

We also found no significant differences in baseline or post-injection levels of glutamate between animals treated with CDPPB alone and those treated with vehicle alone. Since the effects of CDPPB on extracellular glutamate levels have been largely

unexamined, future studies employing other doses of CDPPB, as well as longer or shorter treatment regimens are necessary to further confirm this lack of effect. Other studies have reported that activation of mGluR5 receptors with orthosteric agonists can increase basal or evoked glutamate release from the forebrain and other brain regions (de Novellis et al., 2003; Fazal et al., 2003; Thomas et al., 2000; Pintor et al., 2000; Reid et al., 1999). Furthermore, the slower modulatory effects of mGluR5 PAMs and their allosteric mode of action may contribute to the observed lack of effects of CDPPB alone on extracellular glutamate levels in the mPFC.

The precise neurochemical circuits and signaling mechanisms underlying both the ability of MK-801 to increase extracellular glutamate in the mPFC, and its reversal by CDPPB are currently unknown and require further study. Both NMDA and mGluR5 receptors are predominantly localized to postsynaptic membranes on dendritic spines (Gass & Olive, 2008; Niswender & Conn, 2010; Romano et al., 1995). Thus the effects of ligands on these receptors are not likely to be mediated by actions on presynaptically localized mGluR5 or NMDA receptors, though some investigators have reported evidence of small populations of cortical neurons expressing these receptors on presynaptic terminals (Corlew et al., 2008; Duguid, 2013; Romano et al., 1995). A more likely mechanism is that both CDPPB and MK-801 act on multisynaptic feedback mechanisms that regulate local glutamate levels in the mPFC. For instance, it has been demonstrated that local perfusion of NMDA antagonists into the mPFC does not evoke increases in extracellular glutamate (Lorrain et al., 2003), suggesting that NMDA antagonists act elsewhere in the brain to produce their effects on mPFC extracellular glutamate levels. It has been suggested that NMDA antagonists such as MK-801 inhibit

GABAergic inputs to mPFC glutamate neurons, and thus disinhibit local glutamate transmission (Olney & Farber, 1995; Moghaddam et al., 1997; Krystal et al., 2003; Yonezawa et al., 1998). In support of this suggestion, GABAergic interneurons in subcortical regions such as the limbic cortex and hippocampus appear to be more responsive to NMDA antagonists than cortical pyramidal neurons, indicating that low to moderate doses of MK-801 (Grunze et al., 1996; Li et al., 2002), similar to the dose used in the present study, may act in extra-mPFC regions to influence local GABAergic regulation of glutamatergic transmission in the mPFC. Consistent with this, mGluR5 receptors in the cerebral cortex are predominantly localized to various types of GABAergic interneurons, but are relatively sparse on excitatory pyramidal cells (Kerner et al. 1997). Via their well-characterized facilitating effects on NMDA receptor function (Krystal et al., 2010; Niswender & Conn, 2010; Olive, 2009), mGluR5 PAMs may therefore restore impaired NMDA receptor functionality. Repeated administration of CDPPB has also been reported to increase total levels of NR1 subunits of the NMDA receptor as well as levels of phosphorylated NR1 and NR2B in the frontal cortex (Uslaner et al., 2009), providing another potential mechanism for the ability of mGluR5 PAMs to restore disrupted NMDA functionality.

In summary, we have demonstrated that positive allosteric modulation of mGluR5 receptors prevent the ability of the NMDA receptor antagonist MK-801 to increase extracellular glutamate levels in the mPFC, while showing no evidence of effect when administered alone. The ability of mGluR5 PAMs to reverse disruptions in excitatory transmission in the mPFC is consistent with their ability to reverse pharmacologically induced disruptions in cognitive function, and our findings lend further support to the

notion that mGluR5 PAMs may be novel approaches to restoring cognitive function in neuropsychiatric disorders such as schizophrenia and drug addiction.

CHAPTER 4

EFFECTS OF MGLUR5 POSITIVE AND NEGATIVE ALLOSTERIC MODULATION ON DENDRITIC SPINE MORPHOLOGY IN THE MEDIAL PREFRONTAL CORTEX

Abstract

Rationale Metabotropic glutamate receptors (mGluR5) are highly involved in synaptic plasticity and are linked to proteins which are known to regulate dendritic spine density and spine morphology. However, no studies have examined how the positive or negative allosteric modulation of mGluR5 affects dendritic spine properties. Dendritic spine density and head are positively correlated with efficient synaptic transmission and cognition. Thus, drugs that exert this effect may be potential treatments for disorders that are associated with cognitive impairments.

Objectives The purpose of this study was determine the effects of the positive and negative allosteric modulation of mGluR5 on dendritic spine density and measures of morphology including spine head diameter, length and volume in pyramidal neurons in the mPFC.

Methods Male Sprague-Dawley rats were treated for ten consecutive days with either 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB 20 mg/kg) or 1-(3-chlorophenyl)-3-(3-methyl-5-oxo-4 H-imidazol-2-yl)urea (fenobam 20 mg/kg) or vehicle. Following the final drug or vehicle treatment, brain sections containing the mPFC underwent diolistic labeling and subsequent imaging by confocal laser scanning microscopy.

Results Compared to animals receiving vehicle, administration of fenobam significantly increased spine density and the overall frequency of spines with small (<20 μm) head diameters, decreased the overall frequency of spines with slightly larger (20-40 μm) head diameters, while showing no evidence of effect on the frequency of spines with larger head diameters (>40 μm) of spine length or volume in the mPFC as compared to vehicle. Administration of CDPPB showed no evidence of effect on spine density, and neither treatment had any effect on spine length or volume.

Conclusions These findings highlight functional differences between mGluR5 PAMs and NAMs with regards to their ability to regular structural plasticity in the mPFC.

Introduction

Cognitive impairments play a major role in several debilitating illnesses, such as drug addiction, Parkinson's disease, Alzheimer's disease, and schizophrenia. The extent of cognitive decline is a major determinant of functional outcome for everyday living as cognitive impairments can disrupt a person's ability to maintain employment, relationships, and in some severe cases, appropriate daily hygiene. Cognitive impairments include deficits in working memory, reference memory, attention, set-shifting strategy, verbal fluency, and other aspects of executive functioning (Green et al., 2004; Hahn-Barma et al., 1998; Lawrence et al., 1998a,b; Lees & Smith, 1983; Silver et al., 2003; Snowden et al., 2002; Taylor et al., 1986). A patient's level of cognitive function correlates strongly with treatment retention and compliance. For example, studies in substance abusing populations have shown that treatment dropouts had significantly poorer cognitive functioning than those who completed the treatment

program (Aharonovich et al., 2006). For patients with schizophrenia, cognitive functions were the strongest predictor of ability to properly manage medications (Jeste et al., 2003).

Several cognitive enhancers, known as nootropics, have mechanisms of action that target several different neurotransmitter systems including serotonin, GABA, cholinergic, and glutamate transmission (Brady et al., 2011; Collingridge et al., 2013; Floresco & Jentsch, 2011; Hyman, 2011; Sofuoglu et al., 2013). Increasing attention has been directed towards targeting glutamatergic transmission for cognitive enhancement, as it is one of the primary mediators of synaptic plasticity (Bliss & Collingridge, 1993; Malenka & Nicoll, 1999; Song & Huganir, 2002).

Glutamate is the most prevalent excitatory neurotransmitter in the central nervous system, mediating as much as 70% of both fast and slow excitatory neurotransmission (Olive, 2009). Glutamate receptors are either ligand-gated ion channels (i.e., ionotropic glutamate receptors, or iGluRs) or G-protein coupled receptors (i.e., metabotropic glutamate receptors, or mGluRs). iGluRs are ion channels that are permeable to cations, and function by allowing Na^+ and Ca^{2+} to enter the cell initiating action potentials and activating various intracellular signaling pathways. iGluRs are located on the head of postsynaptic dendritic spines, mediate fast excitatory neurotransmission, and are divided into three different subtypes, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), and kainic acid (KA) (Olive, 2009). mGluRs are located on both presynaptic terminals and postsynaptic dendritic spines, (Conn & Pin, 1997; Hermans & Challiss, 2001; Pin & Duvoisin, 1995). mGluRs mediate slower, modulatory neurotransmission, and based on their intracellular signaling and pharmacological properties, are categorized into three families. Group I mGluRs consists

of mGluR1 and mGluR5 receptors, Group II mGluRs consists of mGluR2 and mGluR3 receptors, and Group III consists of mGluR4, mGluR6, mGluR7 and mGluR8 receptors (Olive, 2009). For the purposes of the present study, we will focus our discussions on Group I mGluRs.

Group I mGluRs primarily utilize G_q/G_{11} signaling mechanisms which activate phosopholipase C to form diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG activates protein kinase C (PKC), while IP_3 exerts further downstream effects by binding to IP_3 receptors on the endoplasmic reticulum releasing intracellular Ca^{2+} which in turn activates other intracellular messengers such as calcium/calmodulin-dependent kinase II (CaMKII). PKC exerts further downstream effects by phosphorylating CREB and other signaling molecules which can eventually lead to altered gene transcription (Hermans & Challiss, 2001). Other intracellular signaling targets of Group I mGluRs include pathways involving $G_{i/o}$ and G_s signaling, as well as the mitogen-activated protein kinase/extracellular signal-related kinase (MAPK/ERK) and mammalian target of rapamycin (mTOR)/p70 S6 kinase pathway, both of which are highly implicated in the regulation of synaptic plasticity (Hou & Klann, 2004; Iacovelli et al., 2002; Li et al., 2007; Page et al., 2006; Saugstad & Ingram, 2008).

Group I mGluRs, particularly mGluR5, are physically linked to NMDA receptors by various scaffolding proteins (such as Homer, Shank and GKAP), and positively modulate NMDA receptor function. Through its activation of PKC, mGluR5 activation also increases the phosphorylation of NMDA receptor subunits, thereby indirectly increasing the probability of NMDA receptor channel opening (Niswender & Conn, 2010). Due to the postsynaptic localization of Group I mGluRs, these receptors primarily

initiate and regulate long-term potentiation (LTP) and long-term depression (LTD) by postsynaptic mechanisms such as potentiation of NMDA receptor function (Anwyl, 1999; Bellone et al., 2008; Gladding et al., 2009). These structural and biochemical interactions allow for the stimulation of group I mGluRs to activate NMDA receptors without inducing excitotoxicity (Albensi, 2007; Hardingham & Bading, 2003).

Positive and negative allosteric modulators (PAMs and NAMs, respectively) are ligands that either facilitate or inhibit receptor functioning in the presence of glutamate binding to the orthosteric binding site. Thus, mGluR5 PAMs and NAMs are more indirect routes for modulating NMDA receptor function while reducing the possibility of adverse side effects such as excitotoxicity. Several studies have examined the effects of the administration of mGluR5 PAMs on cognitive enhancing abilities in impaired and unimpaired cognitive states. For example, pharmacologically induced cognitive deficits have been shown to be reversed by administration of mGluR5 PAMs in several different paradigms, including operant set-shifting tasks (Darrah et al., 2008; Gastambide et al., 2013), spatial alteration tasks (Fowler et al., 2013; Stefani & Moghaddam, 2010), active allothetic place avoidance (Vales et al., 2010), reversal digging paradigms (Gastambide, 2012), social novelty discrimination (Clifton et al., 2013), and novel object recognition (Horio et al., 2013; Reichel et al., 2011). PAM of mGluR5 also reversed induced sucrose impairments (Vardigan et al., 2010) and taste aversion and inhibitory avoidance tasks (Fowler et al., 2011). Notably, mGluR5 PAMs have also been shown to improve spatial and recognition memory abilities in unimpaired animals (Ayala et al., 2009; Balschun et al., 2006; Uslaner et al., 2009; Xu et al., 2013).

In contrast, various studies have shown that mGluR5 NAMs impair cognitive function in otherwise unimpaired animals. mGluR5 NAMs can impair performance on spatial and working memory tasks (Simonyi et al., 2010). Also, mGluR5 knockout (KO) mice show impaired performance in spatial memory tasks as well as acquisition and extinction of conditioned fear (Lu et al., 1997; Xu et al., 2009). However, not all studies have demonstrated cognitive deficits induced by mGluR5 NAMs (Petersen et al., 2002).

Interestingly, mGluR5 NAMs have also been shown to improve aspects of cognitive impairment. Fragile X syndrome (FXS) is the most common inherited form of mental retardation (Gross et al., 2012). FXS is the result of either a deficiency or loss of function of the fragile X mental retardation 1 (FMR1) protein (McLennan et al., 2011). Models of Fragile X syndrome include fragile X mental retardation 1 (FMR1) protein KO mice. FMR1 KO mice display audiogenic and limbic seizures that are characteristic of FXS, and are rescued by treatment with mGuR5 NAMs such as 2-methyl-6-phenylethynyl-pyridine (MPEP) (Yan et al., 2005) and 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridine (CTEP; Michalon et al., 2012). In FXS patients, mGluR5 NAMs such as 1-(3-chlorophenyl)-3-(3-methyl-5-oxo-4 H-imidazol-2-yl)urea (fenobam) have been shown to improve cognitive impairments (Berry-Kravis et al., 2009). As seen in FXS human tissue, FMR1 KO mice have an increased density of immature elongated spines, which is reversed by mGluR5 NAM treatment (Comery et al., 1997; Irwin et al., 1998; Pop et al., 2014).

Spine structure is coordinated with synaptic function and plasticity (Penzes et al., 2011). For example, larger spine sizes are induced by LTP and smaller spine sizes are induced by LTD (Kasai et al., 2010). Spine morphology is experience-dependent and

subtle changes in structure can have robust effects on synaptic function and connectivity in neuronal circuits (Penzez et al., 2011). Larger surface areas of dendritic spine heads allow more receptor insertion which results in more efficient synaptic neurotransmission (Shen et al., 2008). Postmortem studies on patients with schizophrenia show reduced dendritic spine density on pyramidal neurons, particularly in layer 3 neurons, in the dorsolateral prefrontal cortex (DLPFC) (Glantz & Lewis, 2000). This correlates with schizophrenia patients showing reduced activity of this region during cognitive tasks (Tan et al., 2007). FXS syndrome and several other diseases/syndromes (Alzheimer's Disease and Autism Spectrum Disorders (ASD), have severe impaired cognitive functions, correlated with smaller spine size and density in cortical regions (Comery et al., 1997; Hustler et al., 2010; Irwin et al., 1998; Lancor et al., 2007; Pop et al., 2014; Shanker, 2007). The increasing dendritic spine size and density may ameliorate cognitive impairments in various CNS disorders. However, drugs of abuse can induce increases or decreases in dendritic spine density in various brain regions, depending on drug class (i.e., psychostimulants vs. opiates) (Robinson & Kolb, 2004; Gipson et al., 2014).

Few studies have examined the effects of pharmacological manipulation of mGluR5 receptors on dendritic spine plasticity and morphology. Vanderklish and colleagues found that activation of Group I mGluRs increased spine length in hippocampal neurons in vitro, and Chen and colleagues found that embryonic KO mGluR5 mice resulted in increased spine density in the frontal cortex (Chen et al., 2012; Vanderklish et al., 2002). To our knowledge, one study to date examined the effects of mGluR5 PAMs on dendritic spine density and morphology in the frontal cortex, but this study utilized alcohol self-administration and extinction procedures (Gass et al., 2014).

Currently, no study has examined the effects of mGluR5 NAM on dendritic spine density and morphology in the frontal cortex. The objective of this study was to determine the effects of mGluR5 PAM 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB) and mGluR5 NAM fenobam on spine density, length, head diameter and overall volume.

Methods and Materials

Subjects

Subjects were male Sprague-Dawley rats (Harlan Laboratories, Livermore, CA) weighing 250-300 g upon arrival. Rats were pair-housed and had ad libitum access to food and water under a 12 hr reversed light/dark cycle (lights off 7 am) throughout the duration of the study. The vivarium was held at a temperature of 22±1°C, and animals were maintained in accordance with the guidelines described in the 8th edition of the Guide for the Care and Use of Laboratory Animals. All procedures and facilities were approved by the Institutional Animal Care and Use Committee at Arizona State University.

Drugs Treatments

3-cyano-N-1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB) and 1-(3-chlorophenyl)-3-(3-methyl-5-oxo-4H-imidazol-2-yl)urea (fenobam) were synthesized by Chemir Analytical Services (Maryland Heights, MO) according to previously published methods (Jaeschke et al., 2007; Kinney et al., 2005). Each drug was suspended in a vehicle consisting of 10% v/v Tween 80 (Sigma-Aldrich, St. Louis, MO) and

administered at a dose of 20 mg/kg via the subcutaneous (s.c.) route in a volume of 0.5 ml for ten consecutive days. The dose selected for CDPPB and fenobam were based on previous studies by our laboratory and others that have demonstrated behavioral or cognitive effects of these compounds at similar doses (Ayala et al., 2009; Gass et al., 2014; Horio et al., 2013; Stefani & Moghaddam, 2010; Uslaner et al., 2009; Montana et al., 2009; Watterson et al., 2013) and a lack of neurotoxicity in the mPFC (Gass & Olive, 2009), which has recently been described as an adverse effect of higher doses of mGluR5 PAMs (Parmentier-Batteur et al., 2013).

Tissue Preparation

Diolistic labeling of neurons was performed according to previously published procedures (Shen et al., 2008; Gass et al., 2014). Animals were deeply anesthetized with an injection of sodium pentobarbital (150 mg/kg) via the intraperitoneal (i.p.) route and perfused transcardially with 100 mL of phosphate-buffered saline (PBS, pH = 7.4) containing 0.1% w/v heparin followed by 200 mL of 1.5% w/v paraformaldehyde (pH = 7.4) in 0.1 M PBS. After perfusion, brains were extracted and post-fixed in 1.5% paraformaldehyde (PFA) in PBS for one hour at room temperature. Brains were then sectioned on a vibratome (The Vibratome Co., Series 1000, St. Louis, MO) sectioned in 150 μm serial coronal sections through the PFC. Sections were stored in 25 mM PBS at 4˚C until diolistic labeling procedures.

Preparation of DiI-Coated Microparticles

For preparation of dye-coated microparticles, the lipophilic carbocyanine fluorescent dye, 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate, DiI-C_{18}-(3) (DiI, Molecular Probes, Carlsbad, CA) was dissolved in dichloromethane and applied to 100 mg of 1.3-μm diameter tungsten particles (Bio-Rad, Hercules, CA) on a microscope slide. DiI-coated tungsten particles were scraped from the slide and added to 10 ml of 10 mg/ml polyvinylpyrrolidone (PVP; Sigma-Aldrich). The DiI/PVP solution was sonicated and drawn into Tefzel tubing (Bio-Rad) which was pre-coated with 10 mg/ml PVP. Particles were allowed to settle for 30 minutes, after which the PVP solution was carefully withdrawn from the tubing. The tubing was then dried under 0.4 L/min nitrogen gas for 20 min and then cut into small segments and protected from light until use.

Delivery of DiI -Coated Tungsten Particles

A Helios gene gun (Bio-Rad) fitted with a polycarbonate filter (3.0 μm pore size) was used to deliver DiI-coated tungsten particles into the tissue sections via pressurized helium (85 psi). Each tissue section was removed from PBS and diolistically loaded with a single pulse of helium. After labeling, sections were re-suspended in PBS for 24 hours to allow DiI to diffuse into the cell membranes. Next, sections were submerged in a 4% PFA solution for one hour, washed with PBS and mounted on gelatin-coated microscope slides. Sections were covered with anti-fade mounting media to protect against photobleaching, coverslipped, and the edges were sealed with nail polish to prevent dehydration.

Confocal Imaging

A Leica SP5 (Leica Microsystems, Buffalo Grove, IL) with Leica LAS AF software confocal microscope was used to acquire images of DiI labeled neurons. Sections were visualized at 568 nm excitation using a krypton-argon laser. The outline for each labeled neuron was acquired using a 63x/1.4 NA oil immersion objective and an XY pixel resolution of 2048 x 2048 at a frequency of 200 Hz. Each neuron was first scanned at 1.0 μm increments along the z axis to generate an overall image of the neuron and the dendrite to be analyzed, followed by a z-scan at 0.1 μm intervals focusing on the apical dendrite of pyramidal neurons, as characterized by an extensively branched spiny dendrite, as opposed to the shorter basal dendrites. Imaged dendritic segments of identified pyramidal neurons were 70 - 120 μm from the soma. Other criterion for selected dendrites for analysis included being devoid of crossovers from other dendrites, positioned beyond the first branch point, and the dendrite segment analyzed was between 50-80 μm in length. All dendritic segments imaged were located in the prelimbic (PL) or infralimbic (IL) regions of the PFC (N= 6-10 per animal). Localization of cortical layer was noted for each neuron and confirmed by comparison of the position of the neuron analyzed under low (10x) magnification and corresponding sections from a rat brain atlas (Swanson, 1999; Fig 3A).

Quantitation and Analysis of Dendritic Spine Density and Spine Head Morphology

Z-stacks of scanned dendrite segments were reconstructed using Imaris software (Version 7.5, Bitplane Inc., St. Paul, MN). Dendritic shafts and spines were manually traced using the Auto Depth function of the Filament module. Accurate quantification

was achieved using the diameter function. The minimum and maximum values for the dendrite diameter were set at ≥0.3 μm and ≤3.7 μm, respectively, and the fluorescence contrast threshold was set at 1.3 μm. The minimum and maximum values of the segment diameter for dendritic spines were set at ≥0.143 μm and ≤10.5 μm, respectively, and the fluorescence contrast threshold was set at 3 μm. These settings were adequate to distinguish all fluorescent edges which allowed for accurate representation of the shape and size of dendrites and spines analyzed (Shen et al., 2008; Gass et al., 2014). All dendritic and spine tracing were performed by an investigator blind to experimental treatment group. Figure 3B shows a scanned pyramidal neuron with traced dendritic segment and spines.

Data Analyses

Overall differences in each variable (spine density, volume, length, and head diameter) across treatment groups were analyzed using SAS (SAS Institute Inc., Research Triangle Park, NC) using mixed model (SAS Proc Mixed) procedure as we have used previously (Kroener et al., 2012; Gass et al., 2014). The procedure uses a first-order autoregressive covariance matrix across the sequential slices within each rat. Overall tests of significance are determined by Type 3 tests of fixed effects, and post-hoc comparisons are determined by differences in least squares means. Morphological characteristics of dendritic spines (length, volume, and head diameter) were also binned for frequency analyses (Shen et al., 2008), where the number of observations in each bin was expressed as a percent of the total number of observations for that particular variable. The following bin sizes were used: spine volume (0.05 μm^3), length (0.5 μm), and head diameter (0.2

μm). Binned data were analyzed by a two-way ANOVA with bin and treatment group as main factors, followed by Bonferroni post hoc tests using Prism version 5.0 (GraphPad, La Jolla, CA). P-values less than 0.05 were considered statistically significant for all analyses.

Results

We observed a significant effect of treatment on dendritic spine density ($F(2,21)=6.07$, $p<0.01$), such that rats treated with fenobam exhibited significantly increased spine density as compared to rats treated with vehicle (Figure 3C, $p<0.001$). However, there was no evidence of an effect of CDPPB on spine density as compared to vehicle treated animals ($p>0.05$).

We also observed a significant effect of treatment on dendritic spine head diameter ($F(2,21)=6.93$, $p<0.005$), such that rats treated with fenobam exhibited significantly decreased spine density as compared to rats treated with vehicle (Figure 3D, $p<0.005$). Binned frequency analysis revealed that the reduction of spine head diameter by fenobam was accompanied by an increase in frequency of occurrence of spine heads with diameters <20 μm ($p<0.001$) and a decrease in frequency of occurrence of spine heads with diameters between 20 and 40 μm in medium sized spine heads ($p<0.01$). No group differences were observed in the frequency of occurrence of spine heads with diameters >40 μm. In addition, CDPPB showed no evidence of effect on spine head diameter as compared to vehicle treated animals ($p>0.05$).

We found no evidence of effect for treatment on either spine length or volume, either as overall group differences of when data analyzed by binned frequency of occurrence (Figure 3E/F, p's >0.05).

Discussion

The overall findings of this study are that repeated administration of the mGluR5 NAM fenobam increases dendritic spine density in pyramidal neurons of the mPFC, while repeated treatment with the mGluR5 PAM CDPPB showed no evidence of effect on spine density. Analysis of spine head diameters revealed that fenobam treatment produced an overall reduction in spine head diameter, and binned frequency analysis revealed that fenobam treatment increased the frequency of occurrence of spine head diameters <20 μm, decreased the frequency of occurrence of spine head diameters between 20 and 40 μm, while leaving spine head diameters >40 mm unaffected. Suprisingly, CDPPB was without effect on spine density or spine head diameter. Finally, neither CDPPB nor fenobam treatment altered spine volume or length.

Fenobam may increase spine density by acting as an antagonist on the mGluR5 receptor, thus reducing mGluR5 activity. The reduction in mGluR5 activity may cause reduced NMDAR activity, which is critical for normal synaptic function. Therefore, increased spine density may be due to reduced neuronal signaling that may mediate cellular compensatory mechanisms in an effort to return stability to synaptic function. Dendritic spines are plastic and change rapidly as a function of behavior, hormonal status, and synaptic activity (Brandon & Cross, 1982; Horner, 1993; Woolley et al., 1990). mGluR5 activation affects several postsynaptic scaffolding proteins (such as

Homer, GKAP, and Shank) as well as intracellular signaling cascades including PKC and phosphoinositol signaling, all of which influence synaptic maturation and plasticity (Chen et al., 2012). Proper functioning of mGluR5 and NMDAR are essential for neuronal cytoarchitectural maturational processes, including dendritic morphology, axonal arborization (She et al., 2009; Wijetunge et al., 2008), and spine density (Datwani et al., 2002). Studies that support this notion include findings that mGluR5 knockout (KO) mice showed significant increases in spine density in layer IV of the cerebral cortex (Chen et al., 2012), and deletion of NR1 subunit in NMDAR also produces an increase in spine density (Datwani et al., 2002). mGluR5 may indirectly regulate synaptic plasticity mediated by NMDARs (Harney et al., 2006), as inhibition of mGluR5 leads to a reduction of NMDAR currents (Lu et al., 1997). The above studies are consistent with our findings, and overall it appears that decreased mGluR5 and/or NMDAR function causes increased spine density.

Fenobam caused an increase in the frequency of spines with smaller terminal head point diameter and decreased the frequency of spines with medium terminal head point diameters, when compared to CDPPB and vehicle groups. However, CDPPB and vehicle groups showed no significant differences. Spines with smaller terminal head point diameter may be caused by mGluR5-mediated reduced NMDAR activity. Lack of excitatory function could increase the frequency of spines with smaller head size diameter as a result of cellular compensatory mechanisms. In an effort to maintain stable excitatory function, spinogenesis may occur to facilitate neurotransmission. Spine formation is often preceded by the development of filopodium-like or immature/thin spines, which are characterized as having small head diameter (Dailey et al., 1996;

Morest, 1969; Papa et al., 1995; Ramoa et al., 1987; Ulfhake & Cullheim, 1988). Filopodium-like spines have the potential to grow into mature spines and regulate synaptic function (Feng et al., 2002; Grutzendler et al., 2002; Parnass et al., 2000; Trachtenberg et al., 2002). Intracellular proteins that physically link mGluR5 and NMDAR also influence spine morphology. As mentioned earlier, mGluR5 and NMDAR are linked primarily by proteins such as Shank and Homer, but several other post synaptic proteins are involved as well. PSD-95 binding proteins link Shank to NDMAR (Boeckers et al., 1999; Naisbitt et al., 1999; Tu et al., 1999) and Homer binds to Shank, mGluR5 and IP3 (Naisbitt et al., 1999; Tu et al., 1999; Xiao et al., 2000). Through these connections, Shank can link NMDAR and mGluR5 (Sala et al., 2001). Shank promotes the enlargement of spine heads via mechanisms that depend on the post synaptic recruitment and binding of Homer (Lim et al., 1999; Sala et al., 2001). Homer can promote spine enlargement by activating intracellular Ca^{2+} release in response to activation of mGluRs (Tu et al., 1998; Xiao et al., 1998). Relevant to the current study, fenobam acts as an antagonist on mGluR5 and indirectly reduces NMDAR activity, thereby reducing overall intracellular Ca^{2+} levels. This could lead to a disruption of mechanisms that would normally induce spine maturation causing a higher frequency of immature spines. This conclusion is consistent with a study by Chen and colleagues 2012 that found mGluR5 KO mice show significant increases in small spines in layer IV of the cerebral cortex (Chen et al., 2012), and is also consistent with the results of our study that fenobam increases the frequency of spines with smaller terminal head point diameter.

CDPPB resulted in no significant differences on spine head diameter when compared to vehicle. The reasons for this are currently unclear, since mGluR5 PAMs are

known to facilitate the induction of plasticity at hippocampal synapses (Ayala et al., 2009) and alter the expression of NMDA receptor subunits in the mPFC (Uslaner et al., 2009). However, recent published literature has demonstrated that following alcohol self-administration, repeated administration of a similar dose of CDPPB showed no evidence of effect on dendritic spine density or morphology in the mPFC when rats were subjected to forced abstinence in the home cage. Alternatively, a separate group of rats underwent extinction training following alcohol self-administration, CDPPB produced and increase in dendritic spine density, maturity, and AMPA-mediated calcium currents selectively in the infralimbic cortex (Gass et al., 2014). This suggests an interaction between CDPPB and engagement in behaviors known to invoke activity of the mPFC in order to produce discernible changes in spine density or morphology. Finally, the lack of observed effects in spine length, volume, density, or head diameter in CDPPB treated rats are consistent with findings that repeated administration of CDPPB (30 mg/kg) found no evidence of neurotoxicity in the mPFC (Gass & Olive, 2009). This is particularly important in light of recently published evidence that high doses of mGluR5 PAMs can produce neuronal necrosis in the auditory cortex and hippocampus (Parmentier-Batteur et al., 2013).

Both fenobam and CDPPB revealed no significant difference on spine length or spine volume. This may be due to several factors, including short half-life of both drugs, and/or the slower modulatory effects of PAMs and NAMs as well as their allosteric mode of action. CDPPB has a half-life of 4 hrs (Kinney et al., 2005), and fenobam has a half-life of 1 hr (Keck et al., 2013). Animals were treated once daily, thus subsequent studies may detect additional significant group differences by administering drugs twice daily.

It should be noted that while CDPPB and fenobam are both PAMs and NAMs, respectively, they have different postsynaptic effects. CDPPB slowly facilitates mGluR5 activity, while fenobam indirectly blocks all mGluR5 activity (Keck et al., 2013; Kinney et al., 2005). The different effects on mGluR5 activity may explain why significant differences were observed between fenobam and vehicle, but not CDPPB and vehicle, on spine density and spine head diameter. Additionally, spines are highly plastic and undergo continual actin-based motility from seconds to minutes (Fisher et al., 1998; Matus et al., 2000). Because of the dynamic nature of spine morphology, it may be that spines are able to rapidly adapt to drug-induced changes, ultimately resulting in fewer group differences.

In summary, this study showed that negative allosteric modulation of mGluR5 receptors increased spine density and frequency of spines with smaller head diameter, while showing no evidence of effect on spine length or volume in the mPFC. Positive allosteric modulation of mGluR5 receptors were found to show no evidence of effect on spine density, spine head diameter, and spine length or spine volume. Our findings suggest that negative allosteric modulation of mGluR5 may cause neuronal compensatory mechanisms to stabilize synaptic function. However, since there are few other studies on the effects of mGluR5 PAMs and NAMs on dendritic spine density and morphology, future studies utilizing other doses, durations of treatment, and in animal models of neuropsychiatric diseases are clearly warranted.

CHAPTER 5

SUMMARY AND DISCUSSION

Summary of Experiments

Prior studies have shown that mGluR5 PAMs alleviate experimentally induced cognitive impairments in models of schizophrenia in rodents (Darrah et al., 2008; Gilmour et al., 2013; Stefani & Moghaddam, 2010; Vales et al., 2010). The aims of the current studies were to examine the effects of the mGluR5 PAM CDPPB on cognitive function and neurotransmission utilizing an MK-801-induced model of schizophrenia. Additionally, we examined the effects of CDPPB on dendritic spine plasticity, which has been suggested to underlie the cognitive impairments associated with schizophrenia (Glantz & Lewis, 2000; Tan et al., 2007). Experimental results revealed that pretreatment of CDPPB reverses cognitive and neurochemical MK-801-induced effects, but that chronic treatment of CDPPB produced no detectable effects on the structural plasticity of neurons.

Experiment 1 showed that in a delayed matching/non-matching to sample task, CDPPB administration 30 min prior to MK-801 administration was able to prevent cognitive impairments, as compared to simultaneous administration of CDPPB and MK-801, which showed only a slight reversal of impairments. Experiment 2 revealed that chronic administration of MK-801 significantly increases extracellular glutamate levels in the mPFC, and these effects were reversed by chronic CDPPB pretreatment. Experiment 3 showed that CDPPB administered alone caused no significant differences on the structural plasticity of dendritic spines, as measured by dendritic spine density and spine morphology. Interestingly, the mGluR5 NAM fenobam produced significant

increases in dendritic spine density and in the overall frequency of spines with smaller head diameters.

Discussion

Objective of Dissertation

Current medications are inadequate for treating the cognitive impairments observed in schizophrenia. Although the published literature is often contradictory, it appears that there is actually little clinical difference between the two classes of current antipsychotics medications (Green et al., 1997; Jones et al., 2006; Meltzer & McGurk, 1999; Lewis & Lieberman, 2008; Silver et al., 2003; Stroup et al., 2003). Because of the current lack of drug efficacy, additional research and drug development is necessary to further improve the quality of life for patients with schizophrenia.

The intent of this project was to assess the viability of mGluR5 PAMs as a possible novel approach for treating the cognitive impairments observed in patients with schizophrenia. While there are several ligands that act as mGluR5 PAMs, the current studies use the prototypical mGluR5 PAM, CDPPB, to pharmacologically enhance mGluR5 activity. Additionally, all experimental groups utilized a chronic treatment regimen to more closely model daily dosing patterns in humans.

The first experiment of this research project examined the effects of CDPPB on cognitive flexibility using a pharmacological model of schizophrenia. Cognitive flexibility is impaired in patients with schizophrenia and is observed by several behaviors, one being perseveration (Milner, 1963). Patients with schizophrenia have been

shown to perform worse than healthy controls on cognitive flexibility tasks, as measured by rates of perseverative responses (Franke et al., 1992; Milner, 1963). The Wisconsin card sorting task is a neuropsychological test implemented to assess cognitive set-shifting abilities, and is suggested to be an indicator of dorsolateral PFC (DLPFC) function (Franke et al., 1992; Goldberg et al., 1987; Joel et al., 1997). Cognitive set-shifting is thought of as an executive function, and is suggested to involve the integration of planning, response inhibition, working memory and problem solving (Duke & Kaszniak, 2000; Grafman & Litvan, 1999; Stuss & Alexander, 2000). Our first study utilized an operant cognitive set-shifting task that assessed the effects of CDPPB pretreatment on MK-801 induced impaired cognitive flexibility, as measured by rate of correct responses following task reversal. This study incorporated a delayed matching/non-matching-to-sample component, which increased the working memory load, and thus the difficulty of the task (Dudchenko, 2004, Dudchenko et al., 2013). Cognitive flexibility is suggested to be PFC-dependent (Franke et al., 1992; Goldberg et al., 1987; Joel et al., 1997). Therefore, the effects of CDPPB on extracellular glutamate levels in the mPFC were measured in Experiment 2.

Our second experiment assessed the effects of CDPPB when administered alone and as a pretreatment to MK-801 on extracellular glutamate levels in the mPFC. Gray and Roth (2007) posited that hyper-glutamatergic function may lead to hypofunctional NMDAR via excitotoxicity. NMDAR hypofunctioning is a leading hypothesis for the pathophysiology of schizophrenia, and has been suggested to underlie cognitive impairment, as well as the other symptoms of schizophrenia (Olney et al., 1999).

Several published studies have reported that MK-801 increases extracellular glutamate levels in the mPFC, and these effects are reversed by traditional antipsychotics (Adams & Moghaddam, 2001; Lena et al., 2006; Lopez-Gil et al., 2007; Lopez-Gil et al., 2009; Lorrain et al., 2003; Moghaddam et al., 1997; Pietraszek et al., 2009; Roenker et al., 2011; Roenker et al., 2012; Zuo et al., 2006). Therefore, this study assessed the ability of CDPPB to reduce MK-801 induced extracellular glutamate levels, and thus provide further support for the use of mGluR5 PAMs as a novel mechanism for treating the cognitive impairments observed in schizophrenia patients.

Dendritic spine structure is coordinated with synaptic function and plasticity (Penzez et al., 2011). Neuronal structure and morphology in the dorsolateral PFC (DLPFC) have been associated with cognitive function in patients with schizophrenia. During cognitive tasks patients showed reduced activity in the DLPFC (Tan et al., 2007), and postmortem analyses showed reduced dendritic spine density in this region (Glantz & Lewis, 2000). Therefore, increasing dendritic spine density and spine morphology in the mPFC may reduce cognitive impairments. Thus, our final study measured the effects of positive and negative allosteric modulation of mGluR5 manipulation, on the structural plasticity of neurons in the mPFC.

The culmination of these experiments sought to tie together the effects of CDPPB on cognitive impairment, dysregulated glutamate transmission, and structural dendritic plasticity in the mPFC. Together, our findings support the notion of mGluR5 PAMs as novel treatment approaches for cognitive impairments in patients with schizophrenia.

Summary of Results

In experiment 1, animals pretreated with CDPPB 30 min prior to MK-801 showed significant increases in correct responding as compared to animals treated with vehicle/MK-801. Furthermore, pretreatment with CDPPB prior to MK-801 resulted in performance of correct responses that were equivalent to vehicle/vehicle treated animals. Simultaneous treatment with CDPPB and MK-801 produced only partial attenuation of MK-801 induced deficits on set-shifting ability, as compared to vehicle/MK-801 treated animals.

In experiment 2, no significant differences were observed in AUC values for baseline (samples 1-4) between treatment groups. Analyses of post-injection AUC values revealed significant effects of treatment group. Post hoc analyses revealed that post-injection AUC values were significantly elevated in vehicle/MK-801 treated animals as compared to vehicle/vehicle treated animals, indicating that MK-801 produced an elevation in extracellular levels of glutamate in the mPFC. Post-injection AUC values in CDPPB/MK-801 treated animals were significantly decreased as compared to vehicle/MK-801 treated animals, indicating a reversal of MK-801 induced increases in extracellular glutamate by pretreatment with CDPPB. No significant differences were detected in post-injection AUC values between vehicle/vehicle and CDPPB/MK801 treated animals, and no significant differences were detected in Post-injection AUC values between vehicle/vehicle and CDPPB/vehicle treated animals. These latter negative results suggest that administration of CDPPB alone does not alter extracellular levels of glutamate in the mPFC.

In experiment 3, animals treated with the mGluR5 NAM fenobam revealed a significant increase in spine density as compared to animals treated with vehicle. However, CDPPB treatment produced no changes in spine density when compared to vehicle treatment. Animals treated with fenobam showed overall reduced spine head diameters as compared to both vehicle and CDPPB treated animals. Frequency analysis revealed that the reduction of spine head diameter by fenobam was associated with an increase in frequency of the smallest spine heads, and a decrease in medium sized spine heads as compared to vehicle treatment. Animals treated with fenobam or CDPPB produced no significant differences on spine length or spine volume, as compared to vehicle.

Results of primary importance include the ability of mGluR5 PAM to prevent MK-801 induced cognitive impairments, as well as MK-801 induced elevated extracellular glutamate levels. Interestingly, the effects of mGluR5 PAM and NAM treatment on spine density and spine morphology were surprising, as we had expected that CDPPB, rather than fenobam, would increase spine density. Careful interpretation and critical comparison to established literature is necessary to determine the contribution of these results to future research on schizophrenia.

Interpretation of Results: Experiment 1

Experiment 1 demonstrated that pharmacological blockade of NMDA receptors with MK-801 produced deficits in a delayed matching/non-matching-to-sample operant set-shifting task, and these effects were prevented when the mGluR5 PAM CDPPB was administered 30 minutes prior to, but not simultaneously with MK-801. Simultaneous

administration of CDPPB and MK-801 resulted in a partial reversal of MK-801 induced deficits. Our results are consistent with findings from several other studies that administered mGluR5 PAMs to reverse MK-801 induced deficits, but in non-delay based operant tasks. Such paradigms previously studied include: spatial alteration tasks (Fowler et al., 2013); novelty discrimination (Clifton et al., 2013); novel object recognition (Horio et al., 2013; Reichel et al., 2011; Uslaner et al., 2009); and active allothetic place avoidance (Vales et al., 2009). mGluR5 PAMs have also been shown to reverse MK-801 induced impairments in sucrose preference (Vardigan et al., 2010), taste aversion and inhibitory avoidance tasks (Fowler et al., 2011), and alterations in PFC neuronal firing patterns (Lecourtier et al., 2007; Homayoun et al., 2008; Homayoun & Moghaddam, 2010). Notably, these studies did not administer CDPPB as a pretreatment, and drug administration was either acute or subchronic, whereas the current study utilized a chronic treatment regimen. Stefani and Moghaddam (2010) utilized a pretreatment regimen, but they administered MK-801 *prior* to CDPPB. Interestingly, their results were consistent with ours, showing a prevention of MK-801 induced impairments in a spatial maze plus task. However, in this study both treatments were administered acutely, whereas our treatments were administered chronically. It may be possible that the order of ligand administration may become more important when treatment regimen is chronic rather than acute. It should be noted that CDPPB has shown enhancement on spatial and recognition tasks in the absence of any impairment (Ayala et al., 2009; Balschun et al., 2006; Uslaner et al., 2009).

To our knowledge, few previous studies have examined the ability of mGluR5 PAMs to reverse pharmacologically induced cognitive deficits in an operant set-shifting

paradigm (Darrah et al., 2008; Gastembide et al., 2012; Gastembide et al., 2013; Gilmour et al., 2013). Operant-based paradigms offer the advantage of modeling tasks used to assess cognitive flexibility and perseveration in humans, such as the Wisconsin Card Sorting Task (Franke et al., 1992; Goldberg et al., 1987; Joel et al., 1997; Silver et al., 2003). Additionally, operant-based paradigms are PFC-dependent, as is cognitive flexibility (Franke et al., 1992; Goldberg et al., 1987; Joel et al., 1997). Cognitive set-shifting is an executive function that involves attention, inhibition of response, working memory and planning/problem solving, all of which have been associated with DLPFC (Duke & Kaszniak, 2000; Grafman & Litvan, 1999; Stuss & Alexander, 2000). Spatial tasks are hippocampal dependent, and are therefore inappropriate for measuring PFC-dependent cognitive flexibility (Aggleton et al., 1986; Olton & Paras, 1979). Two rodent studies which utilized DMS/DNMS and DMTP tasks found that mPFC lesions produced profound deficits in performance on set-shifting, while hippocampal lesions had no evidence of an effect on the ability to set-shift (Joel et al., 1997; Sloan et al., 2006). Secondly, there is a delay component involved in this task, which increases the working memory load (Dudchenko, 2004, Dudchenko et al., 2013), and therefore the difficulty of the task. Because of these two components, this task has high translational value for assessing cognitive flexibility in humans.

Gilmour and colleagues (2013) trained rats to respond for food in a delayed non-matching-to-position nose poke paradigm. As far as we know, this is the only other study to utilize a delay component with positive findings. However, this study tested the efficacy of the novel mGluR5 PAMs LSN2463359 and LSN2814617 on reversal of set-shifting performance deficits induced by the competitive (closed channel) NMDA

receptor antagonist SDZ 220,581. The efficacy of these mGluR5 PAMs were not assessed against the more widely used non-competitive (open channel) NMDA antagonists such as MK-801 or PCP.

It could be argued that in the present study, the behavioral effects of CDPPB and/or MK-801 following task reversal may have resulted from drug accumulation in the plasma or brain during chronic drug administration prior to task reversal. The plasma elimination half-lives of CDPPB in rats are approximately 4 and 2 hrs, respectively (Kinney et al., 2005; Vezzani et al., 1989), and with chronic administration such an accumulation could indeed occur. However, some observations in the present study argue against this possibility. Should any behavioral effects of CDPPB have carried over from the pre-reversal to the post-reversal phase, these effects would likely have been observed in both the CDPPB/MK-801 and CDPPB 30 min/MK-801. However, this was not the case, since a prevention of MK-801 effects occurred only in animals receiving CDPPB 30 min prior to MK-801.

To further clarify this issue, a second control study was performed administering drug treatments only after task reversal. Initiation of all drug treatments following task reversal did not produce any deficits in acquiring the new task, suggesting a lack of carryover effects of MK-801 from the acquisition task phase to the reversal task phase. These observations are consistent with various bodies of literature suggesting that impaired NMDA receptor function at low to moderate doses does not lead to deficits in initial task learning (Chadman et al., 2006; Harder et al., 1998; Murray et al., 1995; Palencia & Ragozzino, 2004; van der Meulen et al., 2003; van der Staay et al., 2011; Wozniak et al., 1990), but appears to have more deleterious effects on set-shifting, which

result in behavioral and cognitive perseveration (Braff et al., 1991; Egerton et al., 2005; Franke et al., 1992; Goldberg et al., 1987; Green et al., 2004; Silver et al., 2003). Finally, it has previously been demonstrated that long-term cognitive impairing effects of chronically administered MK-801 are predominantly observed at high doses (i.e., 0.2 mg/kg; Li et al., 2011), and not at the dose used in the present study (0.06 mg/kg).Taken together, we can interpret that chronic treatment during the acquisition task phase does not affect the validity of findings from the task reversal phase of the first experiment. Thus, we can conclude that the ability of MK-801 to induce deficits in set-shifting, and its reversal by pretreatment with CDPPB are mediated by continued drug administration following task reversal. Additionally, the effects of CDPPB in reversing induced deficits are consistent with the published literature.

Significance of Findings: Experiment 1

This study has resulted in several significant and novel findings. To our knowledge, this is the first study to show that pretreatment of CDPPB prevents MK-801 induced deficits on perseverative responding in a cognitive set-shifting task. Few studies have utilized an operant based cognitive set-shifting task, which is important as set-shifting is a PFC–dependent task, and only one other study incorporated a delay component, which is important for taxing working memory load. The current study utilized chronic drug administration in order to more closely resemble daily intake patterns that are generally required for therapeutic purposes in humans. The delay component and chronic treatment regimen of this study are two novel attributes that provide increased clinical applicability. Other findings from the current study include

results suggesting that MK-801 does not impair the acquisition of learning, but rather the ability to shift tasks.

CDPPB potentiates mGluR5 function, which indirectly increases NMDAR function and thus counters the NMDAR antagonism by MK-801. One of the leading hypotheses for the pathophysiology of schizophrenia is NMDAR hypofunction, thus MK-801 acting as an NMDAR antagonist may model this proposed pathology. The ability of CDPPB to counteract the effects of MK-801 is significant, as it lends further support for targeting mGluR5 with PAMs as a novel treatment to aid with cognitive impairments associated with schizophrenia.

This study adds novel and significant findings to established research. 1) CDPPB pretreatment prevents deficits in an animal model of schizophrenia. 2) High translational value due to the cognitive set-shifting task utilized in this study, and 3) chronic (42 day) treatment regimen which is more analogous to chronic daily dosing in humans. Due to the above listed findings, this study lends significant support for utilizing PAM of mGluR5 as a novel treatment for the cognitive impairments associated with schizophrenia.

Interpretation of Results: Experiment 2

Experiment 2 showed that pretreatment with CDPPB significantly attenuated MK-801 induced increases in extracellular glutamate in the mPFC. This finding implicates potential neurochemical substrates for the pro-cognitive effects of mGluR5 PAMs. Several literature sources show that CDPPB and other mGluR5 PAMs reverse pharmacologically induced performance deficits in PFC-mediated cognitive tasks such as

set-shifting (Stefani and Moghaddam, 2010; Gilmour et al., 2013; Horio et al., 2013; Darrah et al., 2008; LaCrosse et al., 2014). The published literature that is consistent with our finding that CDPPB reverses MK-801 induced dysfunction include an electrophysiological study, which demonstrated that CDPPB prevents MK801-induced increases in spontaneous activity of pyramidal neurons in the mPFC, and also normalizes MK-801 induced disruption in burst activity of these neurons (Lecourtier et al., 2007). Furthermore, repeated administration of CDPPB has been reported to increase total levels of NR1 subunits of the NMDA receptor as well as levels of phosphorylated NR1 and NR2B in the frontal cortex (Uslaner et al., 2009), providing another potential mechanism for how mGluR5 PAMs are able to reverse disrupted NMDA functionality. Critical evaluation of our results as compared to published literature suggests that PAM of mGluR5 function may be a mechanism by which to reverse disrupted excitatory neurotransmission in the mPFC, which may underlie cognitive impairments in schizophrenia.

Administration of MK-801 significantly increases extracellular glutamate levels in the mPFC. This finding is consistent with several other studies that show acute treatment with MK-801 and other NMDAR antagonists increase extracellular glutamate levels in the mPFC (Adams & Moghaddam, 2001; Lena et al., 2006; Lopez-Gil et al., 2007, 2009; Lorrain et al., 2003; Moghaddam et al., 1997; Pietraszek et al., 2009; Roenker et al., 2011; 2012; Zuo et al., 2006). This is an interesting result, as it seems counter-intuitive that NMDAR antagonists would cause an increase in extracellular glutamate levels. In fact, it has been demonstrated that local perfusion of NMDAR antagonists into the mPFC does not increase extracellular glutamate (Lorrain et al., 2003), which suggests that

NMDAR antagonists act elsewhere in the brain to produce their effects on mPFC extracellular glutamate levels. It has been suggested that NMDAR antagonists such as MK-801 block excitatory input onto GABAergic interneurons in the cerebral, which tonically inhibit mPFC glutamate neurons. Thus, MK-801 may cause disinhibition of glutamate transmission, ultimately leading to the observed increase in extracellular glutamate levels (Olney & Farber, 1995; Moghaddam et al., 1997; Krystal et al., 2003; Yonezawa et al., 1998). In support of this hypothesis, GABAergic interneurons in subcortical regions such as the limbic cortex and hippocampus appear to be more responsive to NMDAR antagonists than cortical pyramidal neurons (Kerner et al., 1997). Additionally, mGluR5 receptors in the cerebral cortex are predominantly localized to various types of GABAergic interneurons and are relatively sparse on excitatory pyramidal cells (Kerner et al., 1997). Via their well-characterized facilitatory effects on NMDAR function (Krystal et al., 2010; Niswender & Conn, 2010; Olive, 2010), mGluR5 PAMs may therefore restore impaired NMDA receptor functionality. Thus, we can interpret that low to moderate doses of MK-801 may act in extra-mPFC regions to influence local GABAergic regulation of glutamatergic transmission in the mPFC (Grunze et al., 1996; Li et al., 2002).

Administration of CDPPB alone produced no detectable effects on extracellular glutamate levels in the mPFC. Since the effects of CDPPB on extracellular glutamate levels have been largely unexamined, future studies employing other doses of CDPPB, as well as longer or shorter treatment regimens are necessary to further confirm this lack of effect. Other studies have reported that activation of mGluR5 receptors with orthosteric agonists can increase basal or evoked glutamate release from the forebrain and other

brain regions (de Novellis et al., 2003; Fazal et al., 2003; Thomas et al., 2000; Pintor et al., 2000; Reid et al., 1999). However, it is possible that the slower modulatory effects of mGluR5 PAMs may contribute to the observed lack of effects of CDPPB on extracellular glutamate levels in the mPFC.

No significant differences in basal levels of extracellular glutamate were observed across all four treatment groups. This is surprising for a few reasons: 1) we utilized a chronic (ten day) treatment regimen which is likely to alter basal levels of glutamatergic transmission, 2) it is well established that acute MK-801 administration results in elevated increased extracellular glutamate levels as shown by our results and several other studies, and 3) Zuo and colleagues (2006) demonstrated that repeated administration of MK-801 daily for seven days actually reduced extracellular levels of glutamate in the mPFC, while acute MK-801 administration increased extracellular glutamate levels, but this study used a very high dose of MK-801 (0.6 mg/kg). Based on our results, as compared to established literature it can be interpreted that our MK-801 dose (0.06 mg/kg) was inadequate to cause the same neuronal adaptations that are required to lower extracellular levels of glutamate in the mPFC. Chronic administration of CDPPB resulted in no evidence of an effect on extracellular basal glutamate levels is consistent with behavioral studies showing that CDPPB lacked evidence of an effect on cognitive tasks without a pre-existing deficit (Darrah et al., 2010), and inconsistent with others showing that CDPPB enhanced task performance on spatial and recognition tasks in the absence of a pre-existing deficit (Ayala et al., 2009; Balschun et al., 2006; Uslaner et al., 2009). However, Experiment 3 of this dissertation is consistent with the current study's findings, showing that chronic administration of CDPPB had no observable

effects on dendritic spine plasticity in the mPFC, which is largely mediated by extracellular glutamate.

The precise neurochemical circuits and signaling mechanisms underlying both the ability of MK-801 to increase extracellular glutamate in the mPFC, and its reversal by CDPPB, are currently unknown and require further study. Both NMDA and mGluR5 receptors are predominantly localized to postsynaptic membranes on dendritic spines (Gass & Olive, 2008; Niswender & Conn, 2010; Romano et al., 1995), and thus the effects of ligands of these receptors are not likely to be mediated by actions on presynaptically localized mGluR5 or NMDA receptors, though some investigators have reported evidence of small populations of cortical neurons expressing these receptors on presynaptic terminals (Corlew et al., 2008; Duguid, 2013; Romano et al., 1995). A more likely mechanism is that both CDPPB and MK-801 act on multisynaptic feedback mechanisms that regulate local glutamate levels in the mPFC.

Based on our results, as compared to the previously published results, we hypothesize that the ability of mGluR5 PAMs to reverse MK-801 induced disruptions in excitatory transmission in the mPFC may underlie the reversal of pharmacologically induced disruptions in cognitive function, as shown in Experiment 1 and other published research. Thus, we hypothesize that mGluR5 PAMs may normalize NMDA antagonist-induced dysregulatedglutamatatergic function in the mPFC.

Significance of Findings: Experiment 2

Of primary significance is our finding that pretreatment with CDPPB significantly attenuated MK-801 induced increases in extracellular glutamate in the mPFC. This is an

important finding that implicates potential neurochemical substrates for the pro-cognitive effects of mGluR5 PAMs. Additionally, our study showed that MK-801 administration increases extracellular glutamate levels in the mPFC. This is consistent with established literature, and furthermore the effects of MK-801 on the glutamate system provide support for the excitotoxicity hypothesis of schizophrenia. This hypothesis suggests that hyperglutamatergic functioning can lead to excitotoxic cell death and cause NMDAR to be hypofunctional (Gray & Roth, 2007). Therefore, two significant outcomes are provided by these findings. First, the ability of CDPPB administration to reverse pharmacologically induced elevated extracellular glutamate levels indicates the potential efficacy for use of mGluR5 PAMs as a possible novel treatment for schizophrenia. Secondly, the effects of MK-801 offer further insight and support to the NMDA hypofunction hypothesis of schizophrenia. Our findings that CDPPB shows no evidence of effect on extracellular glutamate levels is meaningful due to the fact that this is the first study to measure the effects of mGluR5 PAMs on extracellular glutamate using *in vivo* microdialysis procedures.

This study adds novel and significant findings to established research. 1) CDPPB pretreatment is effective at reversing pharmacologically induced elevated extracellular glutamate levels in the mPFC. 2) Administration of CDPPB alone showed no evidence of an effect on extracellular glutamate, and 3) these results inform future research directions, including replication of our own findings, as well as studies to further elucidate the neurochemical circuits and signaling mechanisms underlying both the ability of MK-801 to increase extracellular glutamate in the mPFC, and its reversal by CDPPB, which are currently unknown.

Interpretation of Results: Experiment 3

Repeated treatment with the mGluR5 PAM CDPPB showed no evidence of an effect on spine density, spine head diameter, and spine length or spine volume. These results are surprising since mGluR5 PAMs are known to facilitate the induction of plasticity at hippocampal synapses (Ayala et al., 2009) and alter the expression of NMDA receptor subunits in the mPFC (Uslaner et al., 2009). However, these findings are consistent with published literature. Gass and colleagues (2014) showed that repeated administration of CDPPB at similar doses showed no evidenc of an effect on dendritic spine density or morphology in the mPFC after alcohol self-administration and forced abstinence. Alternatively, a separate group of rats underwent extinction training following alcohol self-administration, and in this group CDPPB produced an increase in dendritic spine density, maturity, and AMPA-mediated calcium currents, selectively in the infralimbic cortex. This suggests and interaction between CDPPB and engagement in behaviors known to invoke activity of the mPFC in order to produce discernible changes in spine density or morphology. Vanderklish and colleagues found that activation of Group I mGluRs increased spine length in hippocampal neurons in vitro (Vanderklish et al., 2002). Behavioral studies offer contradictory findings, such that CDPPB has been shown to reverse induced cognitive impairments (Stefani & Moghaddam, 2010; Vales et al., 2009), provide no evidence of effect on cognitive ability without a deficit (Darrah et al., 2008), and show enhancement on spatial and recognition tasks in the absence of any impairment (Ayala et al., 2009; Balschun et al., 2006; Uslaner et al., 2009). Consistency with comparative literature is difficult to determine as few studies exist. The only other study to measure the effects of CDPPB on dendritic plasticity in the mPFC utilized

alcohol treatment and extinction/abstinence procedures (Gass et al., 2014). The addition of alcohol and extinction training may have had drastic effects on dendritic plasticity, as it is evident that spine number and shape are very responsive to pharmacological manipulation and learning (Brandon & Cross, 1982; Horner, 1993; Woolley et al., 1990). Our study strictly utilized repeated treatment of CDPPB and no other variables were incorporated. From the results of the current study and results from comparative literature, we can interpret that CDPPB may only exert pro-cognitive effects with the influence of either impairment/and or learning factors.

Repeated administration of the mGluR5 NAM fenobam increased dendritic spine density in pyramidal neurons of the mPFC. Analysis of spine head diameters revealed that fenobam treatment produced an overall reduction in spine head diameter, and binned frequency analysis revealed that fenobam treatment increased the frequency of occurrence of spine head diameters <20 μm, and decreased the frequency of occurrence of spine head diameters between 20 and 40 μm, while leaving spine head diameters >40 mm unaffected. To date no study has examined the effect of fenobam on structural plasticity, but we can speculate from published literature that examined similar mechanisms of action.

Fenobam may increase spine density by acting as an antagonist on the mGluR5 receptor, thus reducing mGluR5 activity. The reduction in mGluR5 activity may in turn cause reduced NMDAR activity, which is critical for normal synaptic function. Therefore, the increased spine density may be due to reduced neuronal signaling that may mediate cellular compensatory mechanisms in an effort to return stability to synaptic function. Dendritic spines are plastic and change rapidly as a function of behavior,

hormonal status, and synaptic activity (Brandon & Cross, 1982; Horner, 1993; Woolley et al., 1990). mGluR5 activation affects several postsynaptic scaffolding proteins (such as Homer, GKAP, and Shank) as well as intracellular signaling cascades including PKC and phosphoinositol signaling, all of which influence synaptic maturation and plasticity (Chen et al., 2012). Proper functioning of mGluR5 and NMDAR are essential for neuronal cytoarchitectural maturational processes, including dendritic morphology, axonal arborization (She et al., 2009; Wijetunge et al., 2008), and spine density (Datwani et al., 2002). Studies that support this notion include findings that mGluR5 knockout (KO) mice show significant increases in spine density in layer IV of the cerebral cortex (Chen et al., 2012), and deletion of NR1 subunit in NMDAR also produces an increase in spine density (Datwani et al., 2002). mGluR5 may indirectly regulate the synaptic plasticity mediated by NMDARs (Harney et al., 2006), as inhibition of mGluR5 leads to a reduction of NMDAR currents (Lu et al., 1997). The above studies are consistent with our findings, and overall it appears that decreased mGluR5 and/or NMDAR function causes increased spine density.

Fenobam caused an increase in the frequency of spines with smaller terminal head point diameter and decreased the frequency of spines with medium terminal head point diameters. Spines with smaller terminal head point diameter may be caused by mGluR5-mediated reduced NMDAR activity. Lack of excitatory function could increase the frequency of spines with smaller head size diameter as a result of cellular compensatory mechanisms. In an effort to maintain stable excitatory function, spinogenesis may occur to facilitate neurotransmission. Spine formation is often preceded by the development of filopodium-like or immature/thin spines, which are characterized as having small head

diameter (Dailey et al., 1996; Morest, 1969; Papa et al., 1995; Ramoa et al., 1987; Ulfhake & Cullheim, 1988). Filopodium-like spines have the potential to grow into mature spines and regulate synaptic function (Feng et al., 2002; Grutzendler et al., 2002; Parnass et al., 2000; Trachtenberg et al., 2002). Intracellular proteins that physically link mGluR5 and NMDAR also influence spine morphology. As mentioned earlier, mGluR5 and NMDAR are linked primarily by proteins such as Shank and Homer, but several other post synaptic proteins are involved as well. PSD-95 binding proteins link Shank to NDMAR (Boeckers et al., 1999; Naisbitt et al., 1999; Tu et al., 1999) and Homer binds to Shank, mGluR5 and IP3 (Naisbitt et al., 1999; Tu et al., 1999; Xiao et al., 2000). Through these connections, Shank can link NMDAR and mGluR5 (Sala et al., 2001). Shank promotes the enlargement of spine heads via mechanisms that depend on the post synaptic recruitment and binding of Homer (Lim et al., 1999; Sala et al., 2001). Homer can promote spine enlargement by activating intracellular Ca^{2+} release in response to activation of mGluRs (Tu et al., 1998; Xiao et al., 1998). Relevant to the current study, fenobam acts as an antagonist on mGluR5 receptors and indirectly reduces NMDAR activity, thereby reducing overall intracellular Ca^{2+} levels. This could lead to a disruption of mechanisms that would normally induce spine maturation, causing a higher frequency of immature spines. This conclusion is consistent with a study by Chen and colleagues (2012), which found mGluR5 KO mice to show significant increases in small spines in layer IV of the cerebral cortex, and is also consistent with the results of our study that fenobam increases the frequency of spines with smaller terminal head point diameter.

Both fenobam and CDPPB revealed no significant difference on spine length or spine volume. This may be due to several factors, including short half-life of both drugs,

and/or the slower modulatory effects of PAMs and NAMs, as well as their allosteric mode of action. CDPPB has a half-life of 4 hrs in rats (Kinney et al., 2005), and fenobam has a half-life of 1 hr (Keck et al., 2013). Animals were treated once daily and thus subsequent studies may detect additional significant group differences by administering drugs more frequently.

It should be noted that while CDPPB and fenobam are both PAMs and NAMs, respectively, they have different postsynaptic effects. CDPPB slowly facilitates mGluR5 activity, while fenobam blocks all mGluR5 activity (Keck et al., 2013; Kinney et al., 2005). The different effects on mGluR5 activity may explain why significant differences were observed between fenobam and vehicle, but not CDPPB and vehicle on spine density and spine head diameter. Additionally, spines are highly plastic and undergo continual actin-based motility on a time scale from seconds to minutes (Fisher et al., 1998; Matus et al., 2000). Because of the dynamic nature of spine morphology, it may be that spines are able to rapidly adapt to drug-induced changes, ultimately resulting in fewer group differences.

Thus, we hypothesize that blockade of mGluR5 may cause neuronal compensatory mechanisms in an effort to stabilize synaptic function, and that administration of mGluR5 PAMs may require either a pre-existing deficit or a learning component in order to have an effect on dendritic spine plasticity. However, since there are few other studies on the effects of mGluR5 PAMs and NAMs on dendritic spine density and morphology, future studies utilizing other doses, durations of treatment, and in animal models of neuropsychiatric diseases are clearly warranted before conclusions are made.

Significance of Findings: Experiment 3

Administration of CDPPB produced no evidence of effects on dendritic spine density, spine head diameter or spine length and volume. As mentioned above, it is possible that mGluR5 PAMs require a pre-existing deficit and/or the presence of a learning component in order to exert pro-cognitive effects or effects on dendritic spine plasticity. To our knowledge, the only other study that has examined the effects of mGluR5 PAMs on spine morphology was conducted by Gass and colleagues (2014), who showed that repeated administration of CDPPB following alcohol self-administration had no effect on dendritic spine plasticity in the mPFC in animals undergoing forced abstinence (consistent with the present results), and only exerted an effect when combined with extinction training procedures. Thus, future studies are warranted to determine the effects of CDPPB on dendritic spine plasticity in the mPFC when combined with other behavioral or cognitive tasks.

Our observed effects of fenobam on dendritic spine plasticity may be due to drug-induced reductions in glutamatergic signaling that may induce cellular compensatory mechanisms in an effort to return stability to synaptic function. Dendritic spines are plastic and change rapidly as a function of behavior, hormonal status, and synaptic activity (Brandon & Cross, 1982; Horner, 1993; Woolley et al., 1990). Increases in the frequency of spines with smaller terminal head point diameter may be a result of indirect mGluR5 antagonist-induced NMDAR activity. That is, resulting fenobam-induced increases in the frequency of dendritic spines with small head diamaters may be a result of compensatory efforts by pyramidal neurons to overcome chronic mGluR5 and/or NMDAR inhibition.

This study adds the following novel and significant findings to the field: 1) repeated CDPPB treatment had no significant effect on dendritic spine morphology, possibly a result of the absence of a pre-existing neurochemical deficit and/or incorporation of a learning component that may be necessary to exert any changes in dendritic structure that may accompany pro-cognitive effects of mGluR5 PAMs, 2) repeated fenobam treatment produced a significant increase in dendritic spine density and reduced frequency of dendritic spines with small heads, findings that are consistent with those obtained from mGluR5 KO mice (Chen et al., 2012).

Hypotheses Proved or Disproved: Experiment 1

We hypothesized that mGluR5 PAMs would decrease pharmacologically induced cognitive impairments in a set-shifting task designed to assess perseverative responses as measured by percent correct after rule change. Our specific predictions were: MK-801 administration will cause increased perseverative responses (decreased % correct) following task reversal, but MK-801 simultaneously administered with a cognitive enhancer such as CDPPB will reduce this effect. Also, CDPPB administered as a pretreatment to MK-801 will further reduce perseverative responses.

Our findings support our hypothesis and predictions. Our results show that MK-801 administration caused a significant increase in perseverative responses, as indicated by a decreased percent of correct responses following task reversal. Simultaneous administration of CDPPB and MK-801 partially reduced rates of perseverative responses, as indicated by slight increases in percent of correct responses as compared to rats treated with MK-801 alone. Lastly, administration of CDPPB as a pretreatment (30 min prior) to

MK-801 completely prevented perseverative responses, as indicated by a significant increase in percent correct responses, and no significant difference when compared to the vehicle administered group.

Hypotheses Proved or Disproved: Experiment 2

We hypothesized that mGluR5 PAMs would decrease MK-801 induced increases in extracellular glutamate levels in the mPFC. Our specific predictions were that MK-801 administration will increase extracellular glutamate levels in the mPFC, and CDPPB pretreatment prior to MK-801 administration will reduce that effect. Also, we hypothesized that administration of CDPPB alone will cause a decrease in extracellular glutamate levels in the mPFC. Our hypotheses for effects of drug treatments on basal extracellular glutamate levels in the mPFC were as follows: CDPPB will increase basal glutamate levels, CDPPB/MK-801 will result in no significant differences on basal glutamate levels, and MK-801 will decrease basal glutamate levels.

Our hypothesis was generally supported, but not all of our specific predictions were observed. As expected, MK-801 caused significant increases in extracellular glutamate levels in the mPFC, and pretreatment of CDPPB significantly reduced those effects. Surprisingly, chronic CDPPB treatment produced no significant effects on extracellular glutamate levels, which disproves the predicted hypothesis for this treatment group. Furthermore, our hypotheses on effects of drug treatment on basal glutamate levels were not supported for all treatment groups.

The finding that CDPPB resulted in no significant difference on extracellular glutamate levels was not surprising since our hypothesis was based on the ability of

CDPPB to reverse MK-801 cognitive impairments in behavioral tasks. However, the lack of observed effects of CDPPB alone on extracellular glutamate levels was surprising since mGluR5 receptors are well known to modulate NMDA receptor activity. The most unexpected finding was the lack of significant difference in basal dialysate glutamate levels. We utilized a chronic ten day treatment regimen, and thus expected a difference in basal glutamate levels. Zuo and colleagues (2006) showed that repeated administration of MK-801 reduced extracellular glutamate levels in the mPFC, however they used a very high dose of MK-801 (0.6 mg/kg) compared to our dose (0.06 mg/kg). Thus, the currently utilized dose may be lower than that required to produce significant effects on basal extracellular glutamate levels.

Hypotheses Proved or Disproved: Experiment 3

We hypothesized that potentiation and inhibition of mGluR5 activity would produce opposing effects on dendritic spine density and morphology in the mPFC. Specifically, our predictions were that chronic administration of CDPPB will increase dendritic spine density and alter spine morphology (i.e., increase spine head diameter and/or volume) in the mPFC, while chronic administration of fenobam would decrease dendritic spine density and cause decreases in spine head diameter and spine volume in the mPFC.

Surprisingly, chronic CDPPB treatment resulted in no significant differences in dendritic spine density or any of the spine morphology variables measured as compared to vehicle treated animals. However, chronic fenobam treatment resulted in increased dendritic spine density and produced an overall reduction in spine head diameters.

CDPPB and other mGluR5 PAMs have been well established in the published literature as a cognitive enhancer, and thus we hypothesized that CDPPB may exert its procognitive effects by increasing dendritic spine density and spine morphology. Fenobam, an mGluR5 NAM, was predicted to decrease dendritic spine density and spine morphology. This prediction was due to mGluR5 NAMs indirectly inhibiting NMDAR function, which we thought would have led to decreased synaptic function, and thus eventual spine atrophy/loss.

Study Limitations

The current project has several limitations. Experiment 1 lacked a separate unimpaired control group that administered CDPPB alone (i.e., CDPPB/Vehicle). However, given that the purpose of CDPPB administration was to reverse MK-801 induced set-shifting impairments, a CDPPB/vehicle treatment group was not included. We can speculate that CDPPB/vehicle treated animals would have performed no different than vehicle/vehicle treated animals, as several studies have shown CDPPB treatment alone to provide no evidence of effects. Darrah and colleagues (2008) showed that CDPPB treatment resulted in no significant differences when compared to vehicle treatment in an operant-based cognitive flexibility task, similar to our study. Additionally, this study measured the effects of mGluR5 NAM in the same task, and also found no significant differences when compared to vehicle. A second study also reports CDPPB to show no evidence of effect on cognitive set-shifting alone, and in fact reported that animals treated with CDPPB alone needed more time to finish trials (Stefani & Moghaddam, 2010). Our two other studies also showed CDPPB treatment alone to have

no significant effect on either extracellular glutamate levels or spine density/morphology in the mPFC. Thus, it is plausible that cognitive or other types of deficits are necessary in order for mGluR5 PAMs to produce observable effects on parameters such as extracellular glutamate concentrations. However, in unimpaired animals, mGluR5 PAMs have been shown to have improved spatial and recognition memory abilities (Ayala et al., 2009; Balschun et al., 2006; Uslaner et al., 2009) Therefore, addition of a CDPPB/vehicle group may have provided further interesting results to this study, as well as guiding future research directions.

Experiment 2 utilized *in vivo* microdialysis procedures to measure the effects of CDPPB and MK-801 on extracellular glutamate levels in the mPFC. Microdiaylsis is a time consuming and difficult procedure that frequently is hindered by chronic treatment regimens, troubleshooting of the neurochemical assay, and probe patency issues. Thus, our sample sizes (6-8/group) are not as high as would be optimal for these studies. Adding to the difficulty of this procedure, microdialysis typically measures glutamate from multiple sources, both neurons and glia, and currently it was not possible to determine the source(s) of extracellular glutamate detected by microdialysis procedures (Timmerman & Westerink, 1997). Also, we found no treatment effect on basal levels of extracellular glutamate, which was surprising due to the chronic treatment regimen and known effects of MK-801 on extracellular glutamate in the mPFC. Animals were treated once daily, thus it may be possible that due to the short half-life of CDPPB (4 hrs) and MK-801 (2 hrs) (Kinney et al., 2005; Vezzani et al., 1989) treatments may have already cleared the system upon the morning of sample collection. Therefore, a twice daily

treatment regimen may have resulted in significant treatment differences in basal extracellular glutamate levels.

Experiment 3 assessed the effects of both an mGluR5 PAM and NAM on dendritic spine density and spine morphology. This study was by far the most labor-intensive and time consuming. Because of this, the sample sizes of our treatment groups were limited. We chose to assess the effects of mGluR5 modulation on dendritic plasticity to first determine any significant effects, and if so, add additional treatment groups such as MK-801 alone and CDPPB/MK-801. Unfortunately, this was not possible within a reasonable time frame, and thus the interpretations of our findings are limited to how mGluR5 PAMs or NAMs affects dendritic spine plasticity, which may underlie their effects on cognitive functions. Without these additional groups, we can only speculate as to the effects of mGluR5 PAMs or NAMs on dendritic spine plasticity and how these effects are relevant to schizophrenia-related cognitive impairments. Also, the short half-life of CDPPB (4 hrs.) and fenobam (1 hr.) may have affected the significance of our results (Kinney et al., 2005; Keck et al., 2013). These short half-lives indicate that despite ten daily treatments, the effects observed may be more representative of acute administration, rather than our intentions of chronic administration. A twice daily treatment regimen could more adequately model the effects of chronic treatment.

Suggested Directions for Future Research

In regards to the lack of effects of CDPPB alone on extracellular glutamate levels and dendritic spine density and morphology, it is possible that CDPPB may only exert effects in the presence of either an impairment and/or learning/environmental change.

Gass and colleagues (2014) offer support for this suggestion, demonstrating that animals treated with CDPPB only showed increased dendritic spine density and increased spine head diameter in the mPFC when they underwent extinction training following alcohol self-administration, but not when they underwent forced abstinence in their home cage. Additionally, three behavioral studies showed that CDPPB can have cognitive enhancing effects without prior induction of a cognitive impairment (Ayala et al., 2009; Balschun et al., 2006; Uslaner et al., 2009). Alternatively, Darrah and colleagues (2008) showed CDPPB to provide no evidence of effect on cognition in the presence of a learning/memory component, and in the absence of an induced deficit. Due to these contradicting findings in the literature, future studies on effects of mGluR5 PAMs on extracellular glutamate and dendritic structural plasticity should employ different treatment doses and regimens, as well as inducing a deficit prior to CDPPB administration. In addition, investigating the effects of CDPPB in combination with a learning component in the experimental design, are necessary to further clarify potential mGluR5 PAM cognitive enhancing effects.

Results from our second study showed that MK-801 increases extracellular glutamate in the mPFC, and that CDPPB is able to reverse that effect. Currently the exact neurochemical circuits and signaling mechanisms that underlie these effects are unknown. From previously published literature, we can interpret that the effects of these ligands are not likely mediated by presynaptic localized mGluR5 and NMDAR, as they are predominantly localized to postsynaptic membranes on dendritic spines (Gass & Olive, 2008; Niswender & Conn, 2010; Romano et al., 1995). Alternatively, some investigators have reported evidence of small populations of cortical neurons expressing

these receptors on presynaptic terminals (Corlew et al., 2008; Duguid, 2013; Romano et al., 1995). Taken together, it is more likely that both CDPPB and MK-801 act on multi-synaptic feedback mechanisms, which regulate local glutamate levels in the mPFC. However, this is speculation, and thus further research is necessary to clarify and confirm the precise mechanisms for the effects of both CDPPB and MK-801.

Clinical Applicability

Current antipsychotics are not effective for the treatment of schizophrenia cognitive impairments. These studies provide clinical applicability by means of drug discovery that is focused on testing novel compounds to reduce the cognitive impairments of schizophrenia. mGluR5 PAMs appear to be an effective mechanism for the treatment of cognitive impairments associated with schizophrenia, as shown by studies utilizing animal models of schizophrenia thus far. Experiment 1 offers high translational value, as the task is PFC-dependent and employs a high working memory load. Also, we utilized chronic drug administration to more closely resemble daily intake patterns that are generally required for therapeutic purposes in humans. Thus, the attributes of this study more adequately measure behaviors observed in cognitive impairments (i.e., perseverative responses) in people with schizophrenia, and also reflect a treatment regimen that would most likely be utilized in humans. Adding to the clinical relevance of our results is the type of schizophrenia model used. Disruption in excitatory transmission in the mPFC is a leading hypothesis for the pathophysiology of schizophrenia, which may underlie the cognitive impairments observed in these patients. Results from our studies, and those of others, show that NMDAR antagonists and mGluR5 NAMs dysregulate both

excitatory transmission and dendritic plasticity in the mPFC. Furthermore, our findings that mGluR5 PAMs reduce the effects of NMDAR antagonists support the clinical applicability of these novel compounds as possible treatments for the cognitive deficits in people with schizophrenia.

Culmination of Experimental Findings

Administration of the mGluR5 PAM CDPPB resulted in a reversal of cognitive impairments in an animal model of schizophrenia, as observed by a lack of perseverative responses in a cognitive set-shifting task. To identify a possible neurochemical correlate of the ability of CDPPB to reverse cognitive impairments, the effects of CDPPB on extracellular glutamate levels in the mPFC were measured via *in vivo* microdialysis, as were the effects of this compound on dendritic spine density and morphology in this region. Cognitive impairments associated with schizophrenia may stem from dysfunctional glutamatergic circuitries that ultimately result in reduced PFC function (Olney & Farber, 1995; Olney et al., 1999). NMDAR antagonists such as MK-801 and PCP are excellent animal models for the symptoms of schizophrenia (Jones et al., 2011), and MK-801 has been shown to significantly elevate extracellular glutamate levels (Adams & Moghaddam, 2001; Lena et al., 2006; Lopez-Gil et al., 2007, Lopez-Gil et al., 2009; Lorrain et al., 2003; Moghaddam et al., 1997; Pietraszek et al., 2009; Roenker et al., 2011; Roenker et al., 2012; Zuo et al., 2006). In order to better understand possible mechanisms underlying the ability of CDPPB to reverse MK-801 induced cognitive impairments in behavioral tasks, it was necessary to identify whether CDPPB could also reverse the effects of MK-801 on extracellular glutamate levels in the mPFC in *in vivo*

microdialysis. Administration of CDPPB prior to MK-801 resulted in normalized extracellular glutamate levels in the mPFC, and while these findings are currently only a neurochemical correlate of MK-801 induced cognitive impairments, it is tempting to speculate that CDPPB may exert cognitive enhancing effects by reversing hyperglutamatergic singaling that results from MK-801 administration. Surprisingly, administration of CDPPB alone showed no evidence of effects on extracellular glutamate levels.

To further elucidate possible mechansims involved in the pro-cognitive effects of CDPPB, the effects of an mGluR5 PAM and NAM on dendritic spine density and spine head diameter were examined. Dendritic spine density and spine head size are generally correlated with synaptic function. During LTP induction enlargement of spine heads are observed, and during LTD induction smaller spine heads are observed (Kasai et al., 2010). Patients with schizophrenia have observed spine atrophy and loss of dendritic spine density in the DLPFC, as well as decreased neuronal activity during cognitive tasks, as shown by imaging studies (Glantz & Lewis, 2000; Hirsch et al., 1997; Tan et al., 2007). Since cognitive impairments associated with schizophrenia may be due to loss of neuronal plasticity we hypothesized that CDPPB would cause increased spine density and spine head diameter in the mPFC. Surprisingly, CDPPB showed no evidence of significant effects on dendritic spine density or spine head size in the mPFC. However, the mGluR5 NAM fenobam caused significant increases in dendritic spine density as well as the frequency of spines with smaller head size diameter. These findings demonstrate the need for additional studies on the effects of mGluR5 PAMs on dendritic spine density and morphology as possible neurostructural correlates and mechanisms of the cognition

enhancing effects of these compounds. For example, Gass and colleagues (2014) have shown that CDPPB only increased dendritic spine density and spine head size when testing conditions included a learning component (Gass et al., 2014). Taking this finding into account, and the lack of evidence of effects from administration of CDPPB alone on extracellular glutamate levels in experiment 2, it is reasonable to speculate that in order for CDPPB to exert pro-cognitive effects experimental conditons may need to include either a pre-existing deficit, incorporate a learning component into the testing paradigm, or both. It is also possible that the effects of CDPPB may occur by simply blocking the acitivity of MK-801 on the receptor and not via mechanism of learning or plasticity. Due to findings from Gass and collagues (2014) and behavioral studies showing that CDPPB is able to enhance cognition in the absence of a deficit, but in the presence of learning (Ayala et al., 2009; Balschun et al., 2006; Uslaner et al., 2009; Xu et al., 2013), it is more likely that CDPPB may only enhance cognition when the organism is in a compromised state or when the mPFC circuitries are engaged. Thus, the culmination of results from our studies suggest that potentiation of mGluR5 function is a viable approach for cognitive enhancement, particularly in models of schizophrenia, but that further investigation of the exact mechanisms of effects of mGluR5 PAMs is necessary to make more clear conclusions.

Final Conclusions

The findings of these studies are important as they propose an alternative approach to current treatments for schizophrenia, which are currently unable to relieve the cognitive impairments associated with schizophrenia, and have several adverse side

effects. Our work underscores the importance of assessing novel compounds in paradigms that more adequately measure the cognitive deficits of schizophrenia, and utilize treatment regimens reflective of human daily intake. Additionally, this work illustrates the importance of tying together the effects of novel compounds on several aspects including behavior, neurochemistry and neuronal structure in an effort to better interpret and predict drug efficacy. Based on our findings that CDPPB is effective in reducing impairments in cognition and disrupted neurotransmission in rodent models of schizophrenia, we believe that mGluR5 PAMs may be a novel mechanism for the treatment of cognitive impairments in schizophrenia. Finally, further characterization of mGluR5 and NMDAR interactions may provide valuable insight into the causes of cognitive deficits in patients with schizophrenia. This work will hopefully aid in the development of more efficacious treatments, and offer patients with schizophrenia the opportunity to lead fulfilling lives with alleviation of symptoms in all clinical domains (positive, negative, and cognitive).

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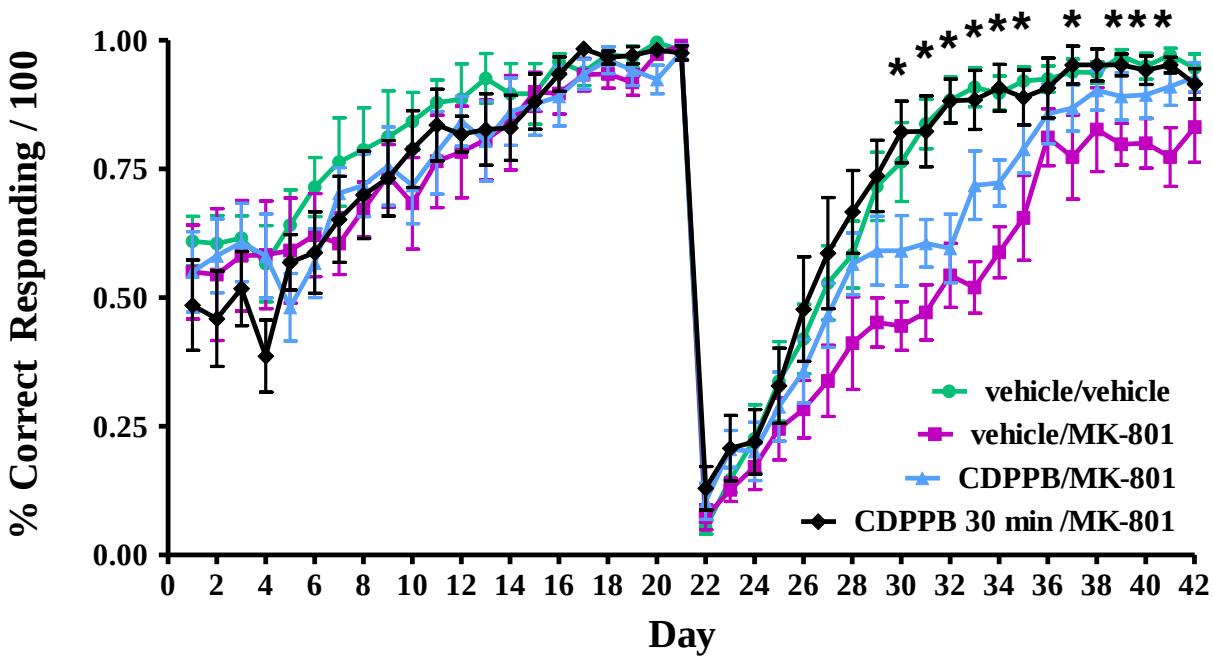

Figure 1A: Performance in DMS/DNMS test for all 4 treatment groups: vehicle/vehicle (n=9), vehicle/MK-801 (n=6), CDPPB/MK-801 (n=7), and CDPPB 30 min/MK-801 (n=9). *indicates $p<0.05$ on days 29-35, 37, 39-41 between groups vehicle/vehicle vs. vehicle/MK-801.

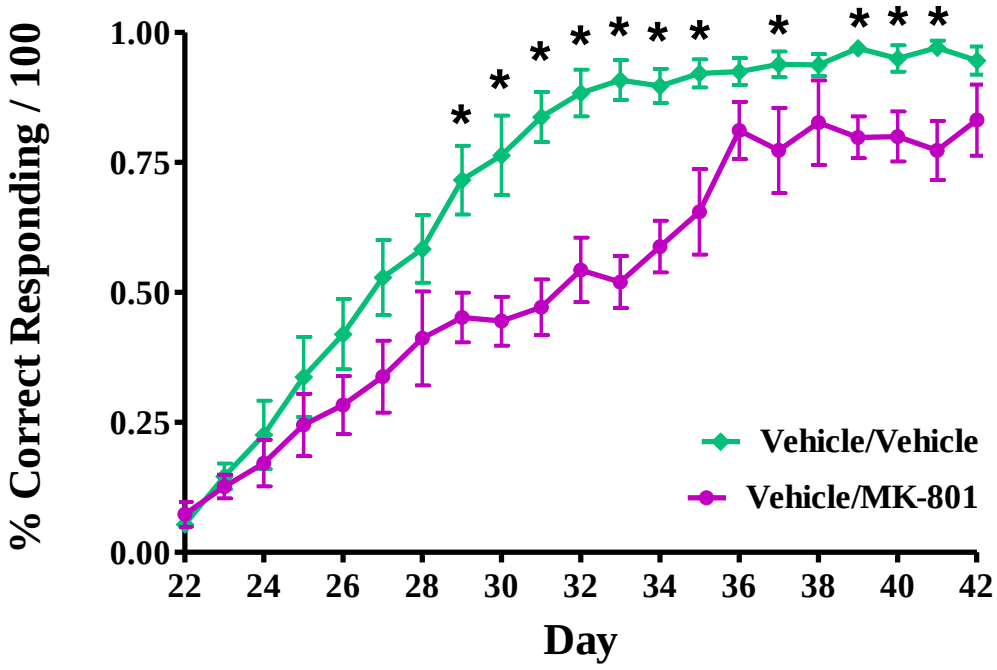

Figure 1B: Re-plotted from figure 1A. Performance in DMS/DNMS test between group vehicle/vehicle and vehicle/MK-801. Administration of MK-801 (0.06 mg/kg) produces a significant deficit in ability to set-shift. *indicates $p<0.05$ on days 29-35, 37, 39-41.

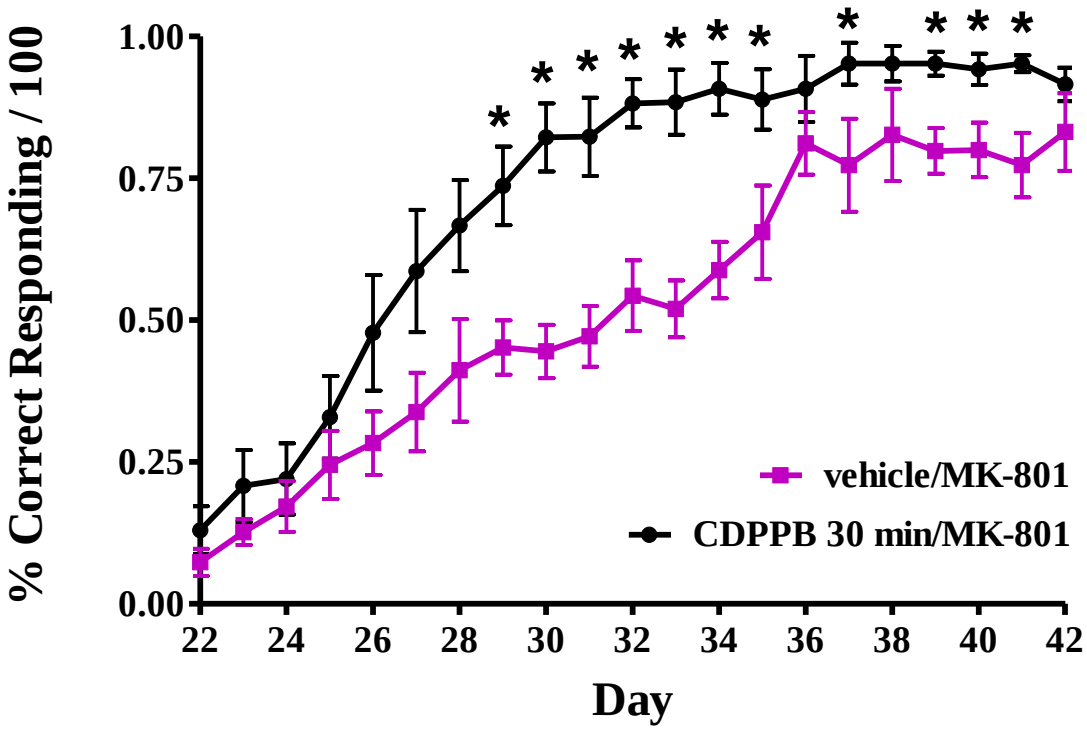

Figure 1C: Re-plotted from figure 1A. Performance in DMS/DNMS test between group CDPPB 30 min/MK-801 and vehicle/MK-801. Administration of MK-801 (0.06 mg/kg) produces a significant deficit in ability to set-shift, which pretreatment with CDPPB (20 mg/kg) reverses. *indicates $p<0.05$ on days 29-35, 37, 39-41.

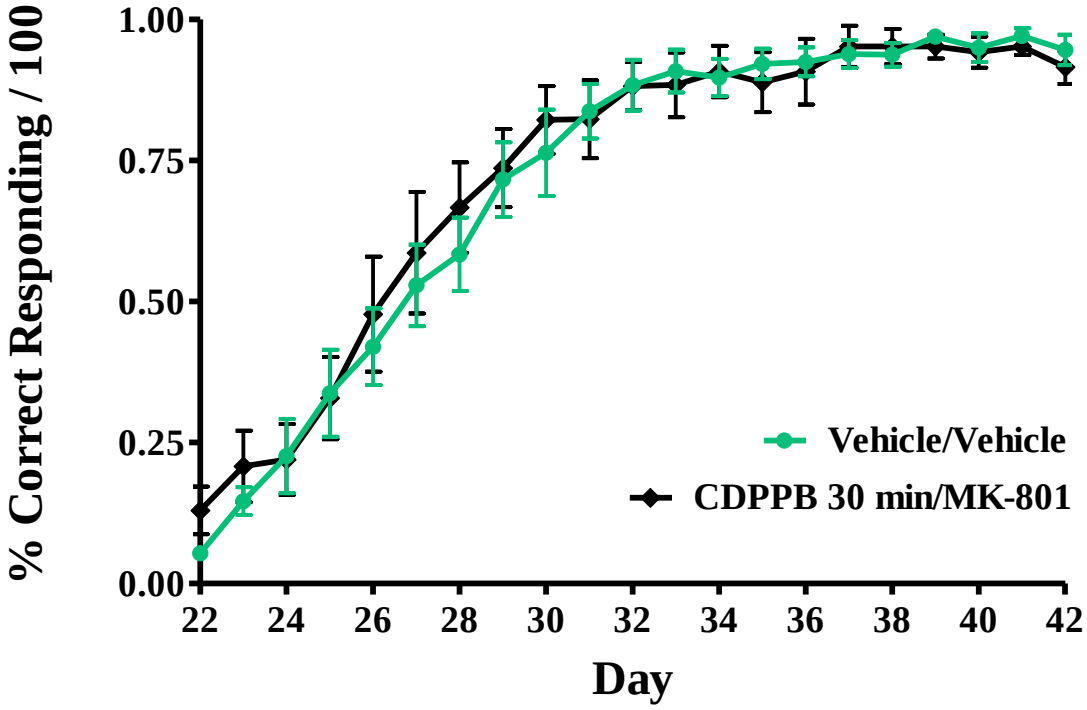

Figure 1D: Re-plotted from figure 1A. No differences in DMS/DNMS performance in unimpaired (vehicle/vehicle) animals and those receiving CDPPB (20 mg/kg) 30 min prior to MK-801 (0.06 mg/kg), ($p>0.05$).

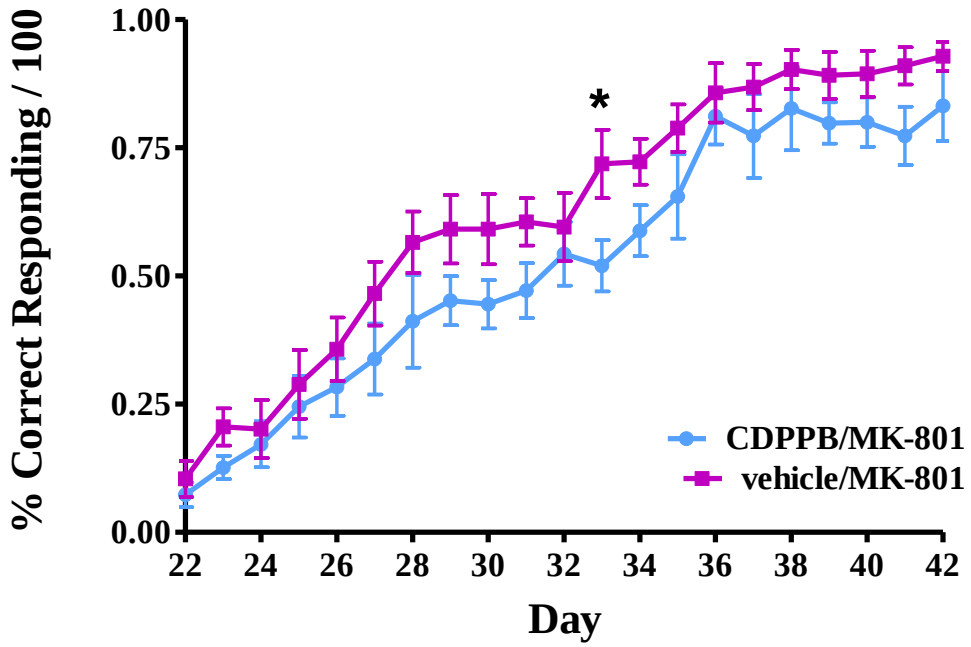

Figure 1E: Re-plotted from figure 1A. Performance in DMS/DNMS test between group CDPPB/MK-801 and vehicle/MK-801. Deficits in set-shifting were alleviated slightly by co-administration of CDPPB (20 mg/kg)/MK-801 (0.06 mg/kg). *indicates $p<0.05$ on day 33, with trends on days 31, 34 and 37 ($p=0.08$, 0.07, and 0.06).

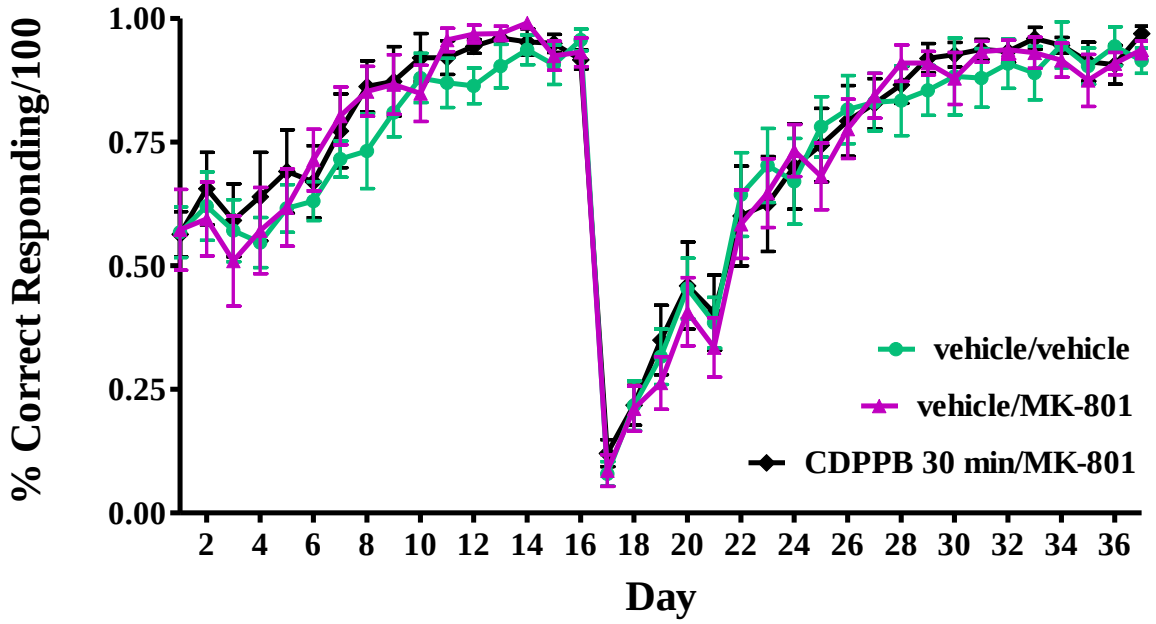

Figure 1F: Performance in DMS/DNMS test between 3 treatment groups CDPPB 30 min/MK-801, vehicle/vehicle, and vehicle/MK-801. No differences in performance in acquiring task reversal when treatments were administered post task-shift ($p>0.05$).

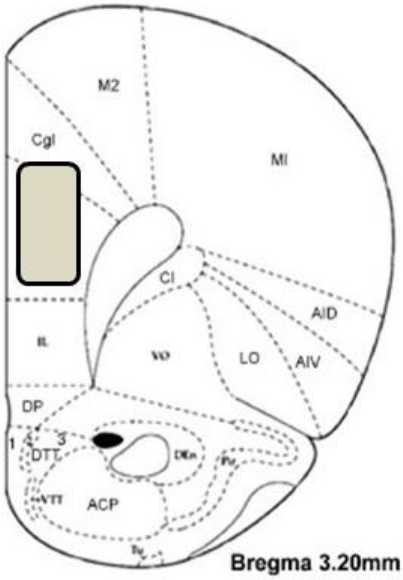

Figure 2A: The gray shaded area represents the area of mPFC where microdialysis probes (2 mm membrane length) were placed in the present study. Drawing adapted from Paxinos & Watson, 2007.

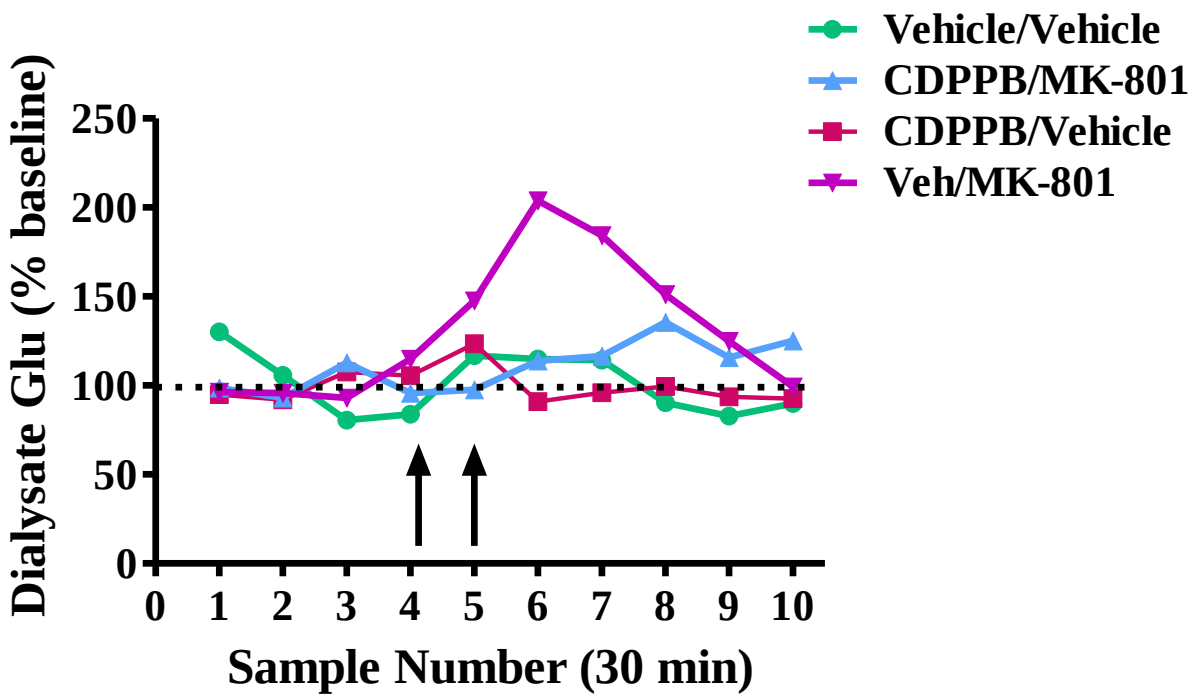

Figure 2B) Effects of chronic administration of CDPPB and/or MK-801 on extracellular glutamate levels in the mPFC. Data points represent mean values within each treatment group and are expressed as percent change from average glutamate levels obtained during collection of four baseline pre-injection samples (30 min each). Error bars are omitted for clarity of data presentation. The first injection (vehicle or CDPPB) was given at arrow 1, followed 30 min later by the second injection, arrow 2 (saline or MK-801).

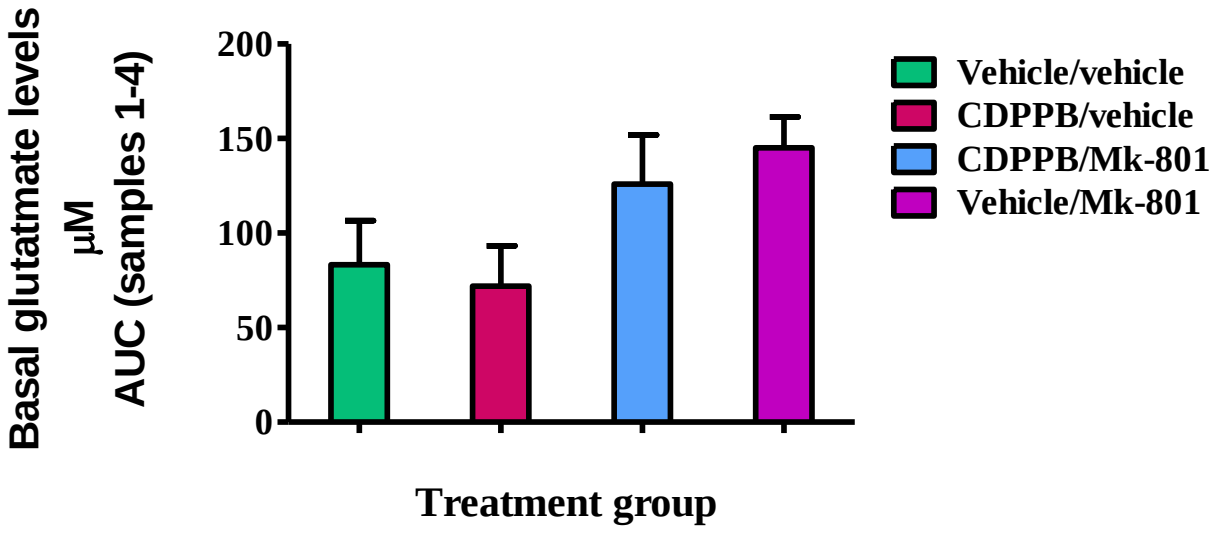

Figure 2C) Effects of chronic administration of CDPPB and/or MK-801 on extracellular glutamate levels in the mPFC. AUC values (based on raw dialysate glutamate content) for pre-injection baseline samples 1-4. Data are presented as mean±SEM, and show no significant group difference ($p>0.05$).

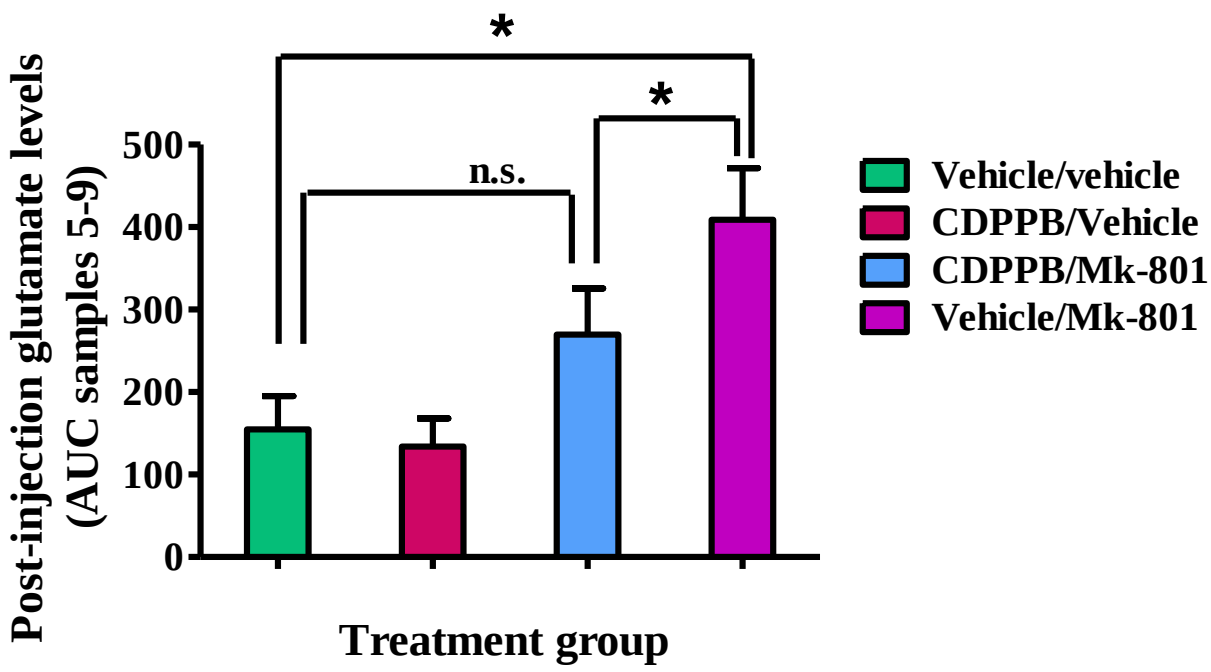

Figure 2D) Effects of chronic administration of CDPPB and/or MK-801 on extracellular glutamate levels in the mPFC. AUC values (based on raw dialysate glutamate content) for post-injection baseline samples 5-9. Data are presented as mean±SEM. * indicates $p<0.05$ between the specified treatment groups. n.s. = not significant.

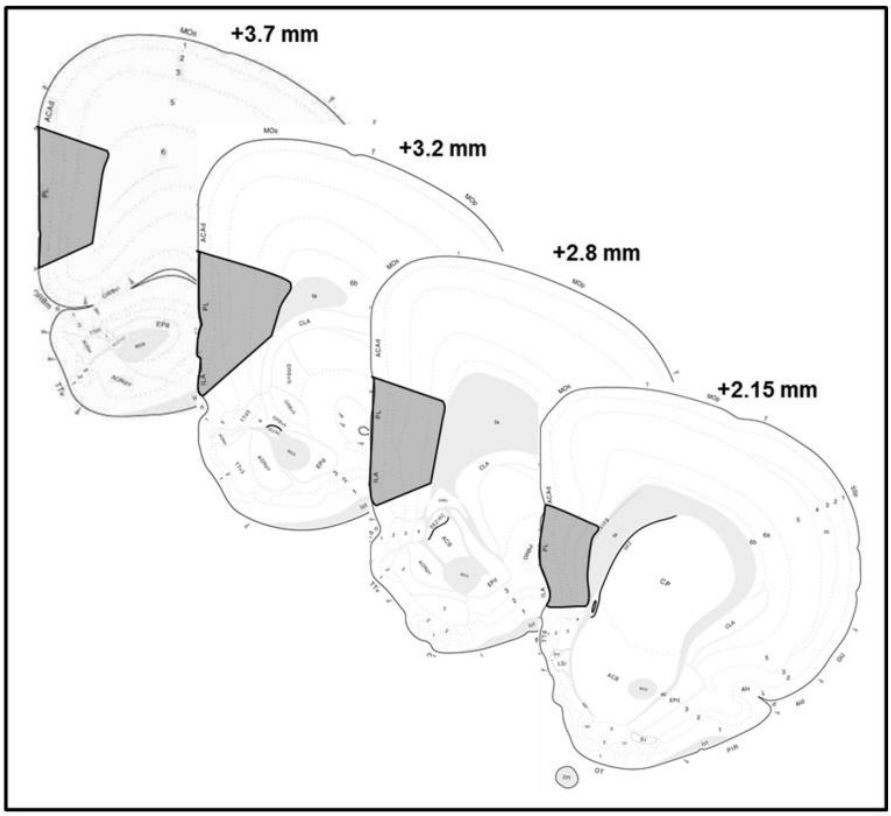

Figure 3A) Location of labeled neurons in the mPFC that were analyzed in the present study. Neurons were chosen for confocal imaging and spine analysis if they were located in the prelimibic (PL) and or infralimbic (ILA) regions. Figure adapted from Swanson (1999).

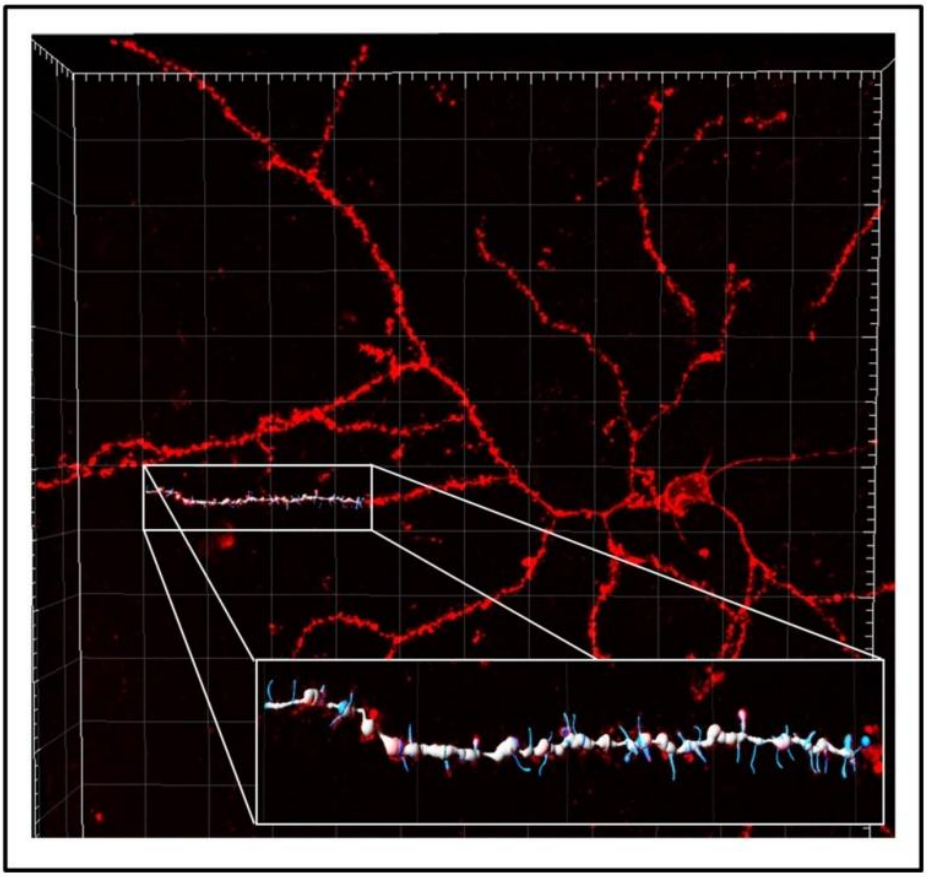

Figure 3B) Representative pyramidal neuron with traced apical dendritic segment and spines (boxed inset).

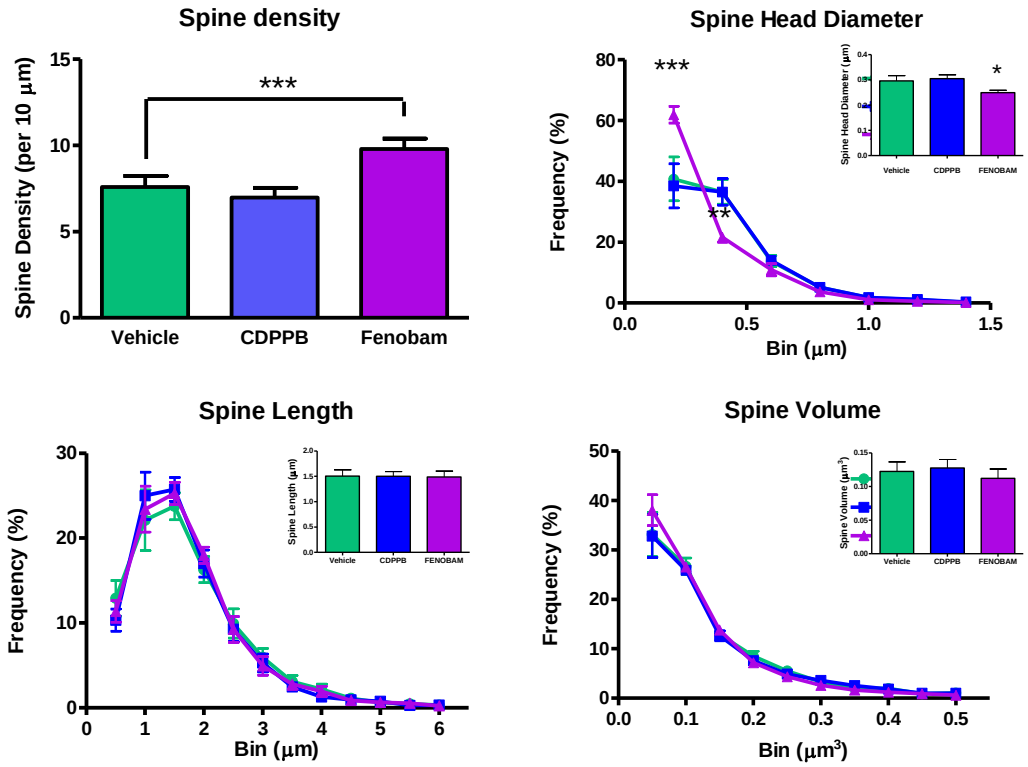

Figure 3C) Compared to vehicle, spine density was increased by fenobam but not by CDPPB (***p<0.001 vs. vehicle). **3D)** Compared to vehicle treated animals, fenobam increased the frequency of smaller spine head diameter. Inset: overall spine head diameter was significantly decreased in rats treated with fenobam compared to vehicle treated rats (*p<0.05).